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(54) Title: DNA EXPRESSION SYSTEMS BASED ON ALPHAVIRUSES

(57) Abstract

Efficient protein production from cloned DNA in animal cells has been hampered by the lack of suitable expression systems. The requirements of such an expression system are (1) to produce functional or immunogenic forms of protein molecules in a wide variety of animal cells, (2) high efficiency and (3) technical simplicity. The present invention is related to a technical solution to this problem. A DNA molecule encoding protein sequences is inserted into engineered variants of the cDNA of a positive stranded RNA virus genome from alphavirus which then, via RNA transcription and transfection into tissue culture cells, is used to produce either chimaeric virus particles for immunization or recombinant virus for protein production. Because of optimized conditions of transfection and the nature of the virus replication the present system combines both simplicity and safety in terms of handling, efficiency in terms of level of protein and RNA production, as well as broad host range.

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DNA EXPRESSION SYSTEMS BASED ON ALPHAVIRUSES

The present invention is related to DNA expression systems based on alphaviruses, which systems can be used to transform animal cells for use in the production of desired products, such as proteins and vaccines, in high yields.

5 The rapid development of biotechnology is to a large extent due to the introduction of recombinant DNA technique, which has revolutionized cell biological and medical research by opening new approaches to elucidate 10 the molecular mechanisms of the cell. With the aid of the techniques of cDNA cloning, large numbers of interesting protein molecules are characterized each year. Therefore, a lot of research activity is today directed to elucidate the relationship between structure 15 and function of these molecules. Eventually this knowledge will increase our possibilities to preserve healthiness and combat diseases in both humans and animals. Indeed, there is today a growing list of new "cloned" protein products that are already used as 20 pharmaceuticals or diagnostics.

In the recombinant DNA approaches to study biological questions, DNA expression systems are crucial elements. Thus, efficient DNA expression systems, which are simple and safe to use, give high yields of the desired 25 product and can be used in a variety of host cells, especially also in mammalian cells, are in great demand.

Many attempts have been made to develop DNA expression systems, which fulfill these requirements. Often, 30 viruses have been used as a source of such systems. However, up to date none of the existing viral expression systems fulfill all these requirements in a satisfying way. For instance, the Baculovirus expression system for cDNA is extremely efficient but can be used only in insect cells (see Reference 1 of the list of cited references; for the sake of convenience, in the following the cited references are only identified by the number they have on said list). As many important molecules will have to be produced and processed in 35

cells of mammalian origin in order for them to become active, this system cannot be used in such cases. Furthermore, the Baculovirus cDNA expression system is not practically convenient for analysis of the relationship between structure and function of a protein because this involves in general the analysis of whole series of mutant variants. Today it takes about 6-8 weeks to construct a single Baculo recombinant virus for phenotype analyses. This latter problem is also true for the rather efficient Vaccinia recombinant virus and other contemporary recombinant virus cDNA expression systems (2,3). The procedure to establish stably transformed cell lines is also a very laborious procedure, and in addition, often combined with very low levels of protein expression.

Hitherto, most attempts to develop viral DNA expression systems have been based on viruses having DNA genomes or retroviruses, the replicative intermediate of the latter being double stranded DNA.

Recently, however, also viruses comprising RNA genomes have been used to develop DNA expression systems.

In EP 0 194 809 RNA transformation vectors derived from (+) strand RNA viruses are disclosed which comprise capped viral RNA that has been modified by insertion of exogenous RNA into a region non-essential for replication of said virus RNA genome. These vectors are used for expression of the function of said exogenous RNA in cells transformed therewith. The RNA can be used in solution or packaged into capsids. Furthermore, this RNA can be used to generate new cells having new functions, i.e. protein expression. The invention of said reference is generally claimed as regards host cells, (+) strand RNA viruses and the like. Nevertheless, it is obvious from the experimental support provided therein that only plant cells have been transformed and in addition only Bromo Mosaic virus, a plant

virus, has been used as transformation vector.

Although it is stated in said reference that it is readily apparent to those skilled in the art to convert any RNA virus-cell system to a useful expression system for exogenous DNA using principals described in the reference, this has not been proven to be true in at least the case of animal cell RNA viruses. The reasons for this seem to be several. These include:

- 10 1) Inefficiencies in transfecting animal cells with in vitro transcribed RNA;
- 15 2) Inefficiency of apparently replication competent RNA transcripts to start RNA replication after commonly used transfection procedures;
- 20 3) The inability to produce high titre stocks of recombinant virus that does not contain any helper virus;
- 25 4) The inability to establish stable traits of transformed cells expressing the function of the exogenous RNA.

In Proc. Natl. Acad. Sci. USA, Vol 84, 1987, pp 4811-4815 a gene expression system based on a member of the Alphavirus genus, viz. Sindbis virus, is disclosed which is used to express the bacterial CAT (chloramphenicol acetyltransferase) gene in avian cells, such as chicken embryo fibroblasts.

Xiong et al., Science, Vol 243, 1989, 1188-1191 also disclose a gene expression system based on Sindbis virus. This system is said to be efficient in a broad range of animal cells. Expression of the bacterial CAT gene in insect, avian and mammalian cells inclusive of human cells is disclosed therein.

Even though it is known from prior art that one

Sindbis

member of the Alphavirus genus, the Sindbis virus, can tolerate insertion and direct the expression of at least one foreign gene, the bacterial chloramphenicol acetyl transferase (CAT) gene, it is evident from the results described that both systems described above are both ineffective in terms of exogenous gene expression and also very cumbersome to use. Hence, neither system has found any usage in the field of DNA expression in animal cells today.

In the first example a cDNA copy of a defective interfering (DI) virus variant of Sindbis virus was used to carry the CAT gene. RNA was transcribed in vitro and used to transfect avian cells and some CAT protein production could be demonstrated after infecting cells with wild-type Sindbis virus. The latter virus provided the viral replicase for expression of the CAT construct. The inefficiency of this system depends on 1) low level of initial DI-CAT RNA transfection (0.05-0.5 % of cells) and 2) inefficient usage of the DI-CAT RNA for protein translation because of unnatural and suboptimal protein initiation signals. This same system also results in packaging of some of the recombinant DI-CAT genomes into virus particles. However, this occurs simultaneously with a very large excess of wild-type Sindbis virus production. Therefore, the usage of this mixed virus stock for CAT expression will be much hampered by the fact that most of the replication and translation activity of the cells infected with such a stock will deal with the wild-type and not with recombinant gene expression.

Much of the same problems are inherent to the other Sindbis expression system described. In this an RNA replication competent Sindbis DNA vector is used to carry the CAT gene. RNA produced in vitro is shown to replicate in animal cells and CAT activity is found. However, as only a very low number of cells are transfected the overall CAT production remains low. Another

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possible explanation for this is that the Sindbis construct used is not optimal for replication. Wild-type Sindbis virus can be used to rescue the recombinant genome into particles together with an excess of wild-type genomes and this mixed stock can then be used to express a CAT protein via infection. However, this stock has the same problems as described above for the recombinant DI system. The latter paper shows also that if virus is amplified by several passages increased titres of the recombinant virus particles can be obtained. However, one should remember that the titre of the wild-type virus will increase correspondingly and the original problem of mostly wild-type virus production remains. There are also several potential problems when using several passages to produce a mixed virus stock. As there is no selected pressure for preservation of the recombinant genomes these might easily 1) undergo rearrangements and 2) become outnumbered by wild-type genomes as a consequence of less efficient replication and/or packaging properties.

Another important aspect of viral DNA expression vectors is use thereof to express antigens of unrelated pathogens and thus they can be used as vaccines against such pathogens.

Development of safe and effective vaccines against viral diseases has proven to be quite a difficult task. Although many existing vaccines have helped to combat the worldwide spread of many infectious diseases, there is still a large number of infectious agents against which effective vaccines are missing. The current procedures of preparing vaccines present several problems: (1) it is often difficult to prepare sufficiently large amounts of antigenic material; (2) In many cases there is the additional hazard that the vaccine preparation is not killed or sufficiently attenuated; (3) Effective vaccines are often hard to produce since there is a major difficulty in presenting the antigenic epitope in

an immunologically active form; (4) In the case of many viruses, genetic variations in the antigenic components results in the evolution of new strains with new serological specificities, which again creates a need for the development of new vaccines.

5 Two types of viral DNA vectors have been developed in order to overcome many of these problems in vaccine production. These either provide recombinant viruses or provide chimaeric viruses. The recombinant viruses contain a wild-type virus package around a recombinant genome. These particles can be used to infect cells which then produce the antigenic protein from the recombinant genome. The chimaeric viruses also contain a recombinant genome but this specifies the production 10 of an antigen, usually as part of a normal virus structural protein, which then will be packaged in progeny particles and e.g. exposed on the surface of the viral spike proteins. The major advantages of these kind of virus preparations for the purpose of being used as a vaccine are 1) that they can be produced in large scale and 2) that they provide antigen in a natural form to the immunological system of the organism. Cells, which have been infected with recombinant viruses, will 15 synthesize the exogenous antigen product, process it into peptides that then present them to T cells in the normal way. In the case of the chimaeric virus there is, in addition, an exposition of the antigen in the context of the subunits of the virus particle itself. Therefore, the chimaeric virus is also called an 20 epitope carrier.

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The major difficulty with these kind of vaccine preparations are, how to ensure a safe and limited replication of the particles in the host without side effects. So far, some success has been obtained with 35 vaccinia virus as an example of the recombinant virus approach (69) and of polio virus as an example of a chimaeric particle (70-72). As both virus variants are

based on commonly used vaccine strains one might argue that they could be useful vaccine candidates also as recombinant respectively chimaeric particles (69-72). However, both virus vaccines are combined with the risk for side effects, even severe ones, and in addition these virus strains have already been used as vaccines in large parts of the population in many countries.

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As is clear from the afore mentioned discussion there is much need to develop improved DNA expression systems both for an easy production of important proteins or polypeptides in high yields in various kinds of animal cells and for the production of recombinant viruses or chimaeric viruses to be used as safe and efficient vaccines against various pathogens.

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Thus, an object of the present invention is to provide an improved DNA expression system based on virus vectors which can be used both to produce proteins and polypeptides and as recombinant virus or chimaeric virus, which system offers many advantages over prior art.

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To that end, according to the present invention there is provided an RNA molecule derived from an alphavirus RNA genome and capable of efficient infection of animal host cells, which RNA molecule comprises the complete alphavirus RNA genome regions, which are essential to replication of the said alphavirus RNA, and further comprises an exogenous RNA sequence capable of expressing its function in said host cell, said exogenous RNA sequence being inserted into a region of the RNA molecule which is non-essential to replication thereof.

Alphavirus is a genus belonging to the family Togaviridae having single stranded RNA genomes of positive polarity enclosed in a nucleocapsid surrounded by an envelope containing viral spike proteins.

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The Alphavirus genus comprises among others the Sindbis virus, the Semliki Forest virus (SFV) and the Ross River virus, which are all closely related.

According to a preferred embodiment of the invention, the Semliki Forest virus (SFV) is used as the basis of the DNA expression system.

The exogenous RNA sequence encodes a desired genetic trait, which is to be conferred on the virus or the host cell, and said sequence is usually complementary to a DNA or cDNA sequence encoding said genetic trait. Said DNA sequence may be comprised of an isolated natural gene, such as a bacterial or mammalian gene, or may constitute a synthetic DNA sequence coding for the desired genetic trait i.e. expression of a desired product, such as an enzyme, hormone, etc. or expression of a peptide sequence defining an exogenous antigenic epitope or determinant.

If the exogenous RNA sequence codes for a product, such as a protein or polypeptide, it is inserted into the viral RNA genome replacing deleted structural protein encoding region(s) thereof, whereas a viral epitope encoding RNA sequence may be inserted into structural protein encoding regions of the viral RNA genome, which essentially do not comprise deletions or only have a few nucleosides deleted.

The RNA molecule can be used per se, e.g. in solution to transform animal cells by conventional transfection, e.g. the DEAE-Dextran method or the calcium phosphate precipitation method. However, the rate of transformation of cells, and, thus the expression rate can be expected to increase substantially if the cells are transformed by infection with infectious viral particles. Thus, a suitable embodiment of the invention is related to an RNA virus expression vector comprising the RNA molecule of this invention packaged into infectious particles comprising the said RNA within the alphavirus nucleocapsid and surrounded by the membrane including the alphavirus spike proteins.

The RNA molecule of the present invention can be packaged into such particles without restraints pro-

vided that it has a total size corresponding to the wild type alphavirus RNA genome or deviating therefrom to an extent compatible with package of the said RNA into the said infectious particles.

5 These infectious particles, which include recombinant genomes packaged to produce a pure, high titre recombinant virus stock, provides a means for exogenous genes or DNA sequences to be expressed by normal virus particle infection, which as regards transformation degree, is much more efficient than RNA transfection.

10 According to a suitable embodiment of the invention such infectious particles are produced by cotransfection of animal host cells with the present RNA which lacks part of or the complete region(s) encoding the structural viral proteins together with a helper RNA molecule transcribed in vitro from a helper DNA vector comprising the SP6 promoter region, those 5' and 3' regions of the alphavirus cDNA which encode cis acting signals needed for RNA replication and the region encoding the viral structural proteins but lacking essentially all of the nonstructural virus proteins encoding regions including sequenses encoding RNA signals for packaging of RNA into nucleocapsid particles, and culturing the host cells.

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35 According to another aspect of the invention efficient introduction of the present RNA into animal host cells can be achieved by electroporation. For example, in the case of Baby Hamster Kidney (BHK) cells a transformation degree of almost 100 % has been obtained for the introduction of an RNA transcript derived from SFV cDNA of the present invention. This makes it possible to reach so high levels of exogenous protein production in every cell that the proteins can be followed in total cell lysates without the need of prior concentration by antibody precipitation.

By electroporation, it is also possible to obtain a high degree of cotransfection in the above process for

5 production of infectious particles comprising packaged RNA of the present invention. Essentially all animal cells will contain both the present RNA molecule and the helper RNA molecule, which leads to a very efficient trans complementation and formation of infectious particles. A pure recombinant virus stock, consisting of up to 10^9 - 10^{10} infectious particles, can be obtained from 5×10^6 cotransfected cells after only a 24 h incubation. Furthermore, the so obtained virus stock is 10 very safe to use, since it is comprised of viruses containing only the desired recombinant genome, which can infect host cells but can not produce new progeny virus.

15 Theoretically, a regeneration of a wild-type virus genome could take place when producing the recombinant virus in the cotransfected cells. However, the possibility to avoid spread of such virus can be eliminated by incorporating a conditionally lethal mutation into the structural part of the helper genome. Such a mutation is described in the experimental part of this 20 application. Thus, the virus produced with such a helper will be noninfectious if not treated *in vitro* under special conditions.

25 The technique of electroporation is well known within the field of biotechnology and optimal conditions can be established by the man skilled in the art. For instance, a BioRad Gene pulser apparatus (BioRad, Richmond, CA, USA) can be used to perform said process.

30 The RNA molecule of the present invention is derived by *in vivo* or *in vitro* transcription of a cDNA clone, originally produced from an alphavirus RNA and comprising an inserted exogenous DNA fragment encoding a desired genetic trait.

35 Accordingly, the present invention is also related to a DNA expression vector comprising a full-length or partial cDNA complementary to alphavirus RNA or parts thereof and located immediately downstream of the SP6

RNA polymerase promoter and having a 5'ATGG, a 5'GATGG or any other 5' terminus and a TTTCCA₆₉ACTAGT or any other 3' terminus.

According to one aspect of the present invention portions of the viral cDNA are deleted, the deletions comprising the complete or part of the region(s) encoding the virus structural proteins, and the vector further comprises an integrated polylinker region, which may correspond to BamHI-SmaI-XmaI, inserted at a location which enables an exogenous DNA fragment encoding a foreign polypeptide or protein to be inserted into the vector cDNA for subsequent expression in an animal host cell.

According to another aspect of this invention, the vector is comprised of full-length cDNA wherein an exogenous DNA fragment encoding a foreign epitopic peptide sequence can be inserted into a region coding for the viral structural proteins.

It is appreciated that this cDNA clone with its exogenous DNA insert is very efficiently replicated after having been introduced into animal cells by transfection.

A very important aspect of the present invention is that it is applicable to a broad range of host cells of animal origin. These host cells can be selected from avian, mammalian, reptilian, amphibian, insect and fish cells. Illustrative of mammalian cells are human, monkey, hamster, mouse and porcine cells. Suitable avian cells are chicken cells, and as reptilian cells viper cells can be used. Cells from frogs and from mosquitoes and flies (*Drosophila*) are illustrative of amphibian and insecticidal cells, respectively. A very efficient virus vector/host cell system according to the invention is based on SFV/BHK cells, which will be discussed more in detail further below.

However, even though a very important advantage of the present DNA expression vector is that it is very

efficient in a broad variety of animal cells it can also be used in other eucaryotic cells and in pro-caryotic cells.

5 The present invention is also related to a method to produce transformed animal host cells comprising transfection of the cells with the present RNA molecule or with the present transcription vector comprised of cDNA and carrying an exogenous DNA fragment. According to a suitable embodiment of the invention, transfection is produced by the above mentioned electroporation method, a very high transfection rate being obtained.

10 A further suitable transformation process is based on infection of the animal host cells with the above mentioned infectious viral particles comprising the present RNA molecule.

15 The transformed cells of the present invention can be used for different purposes.

20 One important aspect of the invention is related to use of the present transformed cells to produce a polypeptide or a protein by culturing the transformed cells to express the exogenous RNA and subsequent isolation and purification of the product formed by said expression. The transformed cells can be produced by infection with the present viral particles comprising exogenous RNA encoding the polypeptide or protein as mentioned above, or by transfection with an RNA transcript obtained by in vitro transcription of the present DNA vector comprised of cDNA and carrying an exogenous DNA fragment coding for the polypeptide or the protein.

25 30 Another important aspect of the invention is related to use of the present transformed cells for the production of antigens comprised of chimaeric virus particles for use as immunizing component in vaccines or for immunization purposes for in vivo production of immunizing components for antisera production.

35 Accordingly, the present invention is also related to an antigen consisting of a chimaeric alphavirus having

an exogenous epitopic peptide sequence inserted into its structural proteins.

Preferably, the chimaeric alphavirus is derived from SFV.

5 According to a suitable embodiment, the exogenous epitopic peptide sequence is comprised of an epitopic peptide sequence derived from a structural protein of a virus belonging to the immunodeficiency virus class inclusive of the human immunodeficiency virus types.

10 A further aspect of the invention is related to a vaccine preparation comprising the said antigen as immunizing component.

15 In said vaccine the chimaeric alphavirus is suitably attenuated by comprising mutations, such as the conditionally lethal SFV-mutation described before, amber (stop codon) or temperature sensitive mutations, in its genome.

20 For instance, if the chimaeric virus particles containing the afore mentioned conditional lethal mutation in its structural proteins (a defect to undergo a certain proteolytical cleavage in host cell during morphogenesis) is used as a vaccine then this is first activated by limited proteolytic treatment before given to the organism so that it can infect recipient cells.
25 New chimaeric particles will be formed in cells infected with the activated virus but these will again be of the lethal phenotype and further spread of infection is not possible.

30 The invention is also concerned with a method for the production of the present antigen comprising
a) in vitro transcription of the cDNA of the present DNA vector carrying an exogenous DNA fragment encoding the foreign pitopic peptide sequence and transfection of animal host cells with the produced RNA transcript,
35 or
b) transfection of animal host cells with the said cDNA of the above step a),

culturing the transfected cells and recovering the chimaeric alphavirus antigen. Preferably, transfection is produced by electroporation.

Still another aspect of the invention is to use a recombinant virus containing exogenous RNA encoding a polypeptide antigen for vaccination purpose or to produce antisera. In this case the recombinant virus or the conditionally lethal variant of it is used to infect cells *in vivo* and antigen production will take place in the infectious cells and used for antigen presentation to the immunological system.

According to another embodiment of the invention, the present antigen is produced in an organism by using *in vivo* infection with the present infectious particles containing exogenous RNA encoding an exogenous epitopic peptide sequence.

In the following, the present invention will be illustrated more in detail with reference to the Semliki Forest virus (SFV), which is representative for the alphaviruses. This description can be more fully understood in conjunction with the accompanying drawings in which:

Fig. 1 is a schematic view over the main assembly and disassembly events involved in the life cycle of the Semliki Forest virus, and also shows regulation of the activation of SFV entry functions by p62 cleavage and pH;

Fig. 2 illustrates the use of translocation signals during synthesis of the structural proteins of SFV; top, the gene map of the 26S subgenomic RNA; middle, the process of membrane translocation of the p62, 6K and E1 proteins; small arrows on the luminal side denote signal peptidase cleavages; at the bottom, the characteristics of the three signal peptides are listed;

Fig. 3 shows features that make SFV an excellent

choice as an expression vector;

Fig. 4 A-C show the construction of full-length infectious clones of SFV; Fig. 4A shows a schematic restriction map of the SFV genome; primers used for initiating cDNA synthesis are indicated as arrows; and the cDNA inserts used to assemble the final clone are showed as bars; Fig. 4B shows plasmid pPLH211, i.e. the SP6 expression vector used as carrier for the full-length infectious clone of SFV, and the resulting plasmid pSP6-SFV4; Fig. 4C shows the structure of the SP6 promoter area of the SFV clone; the stippled bars indicate the SP6 promoter sequence, and the first nucleotide to be transcribed is marked by an asterisk; underlined regions denote authentic SFV sequences;

Fig. 5 shows the complete nucleotide sequence of the pSP6-SFV4 RNA transcript as DNA (U = T) and underneath the DNA sequence, the amino acid sequence of the non-structural polyprotein and the structural polyprotein;

Fig. 6 shows an SFV cDNA expression system for the production of virus after transfection of in vitro made RNA into cells;

Fig. 7 shows the construction of the SFV expression vectors pSFV1-3 and of the Helper 1;

Fig. 8 shows the polylinker region of SFV vector plasmids pSFV1-3; the position of the promoter for the subgenomic 26S RNA is boxed, and the first nucleotide to be transcribed is indicated by an asterisk;

Fig. 9 is a schematic presentation of in vivo packaging of pSFV1-dhfr RNA into infectious particles using helper trans complementation; (dhfr means di-hydrofolate reductase)

Fig. 10 shows the use of trypsin to convert p62-containing noninfectious virus particles to infectious particles by cleavage of p62 to E2 and E3;

Fig. 11 shows the expression of heterologous proteins in BHK cells upon RNA transfection by electroporation; and

Fig. 12 shows in its upper part sequences encompassing the major antigenic site of SFV and the in vitro made substitutions leading to a BamHI restriction endonuclease site, sequences spanning the principal neutralizing domain of the HIV gp120 protein, and the HIV domain inserted into the SFV carrier protein E2 as a BamHI oligonucleotide; and its lower part is a schematic presentation of the SFV spike structure with blow-ups of domain 246-251 in either wild type or chimaeric form.

The alphavirus Semliki Forest virus (abbreviated SFV in the following text) has for some 20 years been used as model system in both virology and cell biology to study membrane biosynthesis, membrane structure and membrane function as well as protein-RNA interactions (4, 5). The major reason for the use of SFV as such a model is due to its simple structure and efficient replication.

With reference to Fig. 1-3, in the following the SFV and its replication are explained more in detail. In essential parts, this disclosure is true also for the other alphaviruses, such as the Sindbis virus, and many of the references cited in this connection are indeed directed to the Sindbis virus. SFV consists of an RNA-containing nucleocapsid and a surrounding membrane composed of a lipid bilayer and proteins, a regularly arranged icosahedral shell of a protein called C protein forming the capsid inside which the genomic RNA is packaged. The capsid is surrounded by the lipid bilayer that contains three proteins called E1, E2, and E3. These so-called envelope proteins are glycoproteins and their glycosylated portions are on the outside of the lipid bilayer, complexes of these proteins forming the "spikes" that can be seen in electron micrographs to project outward from the surface of the virus.

The SFV genome is a single-stranded 5'-capped and 3'-polyadenylated RNA molecule of 11422 nucleotides (6,7).

It has positive polarity, i.e. it functions as an mRNA, and naked RNA is able to start an infection when introduced into the cytoplasm of a cell. Infection is initiated when the virus binds to protein receptors on the host cell plasma membrane, whereby the virions become selectively incorporated into "coated pits" on the surface of the plasma membrane, which invaginate to form coated vesicles inside the cell, whereafter said vesicles bearing endocytosed virions rapidly fuse with organelles called endosomes. From the endosome, the virus escapes into the cell cytosol as the bare nucleocapsid, the viral envelope remaining in the endosome. Thereafter, the nucleocapsid is "uncoated" and, thus, the genomic RNA is released. Referring now to Fig. 1, infection then proceeds with the translation of the 5' two-thirds of the genome into a polyprotein which by self-cleavage is processed to the four nonstructural proteins nsP1-4 (8). Protein nsP1 encodes a methyl transferase which is responsible for virus-specific capping activity as well as initiation of minus strand synthesis (9, 10); nsP2 is the protease that cleaves the polyprotein into its four subcomponents (11, 12); nsP3 is a phosphoprotein (13, 14) of as yet unknown function, and nsP4 contains the SFV RNA polymerase activity (15, 16). Once the nsP proteins have been synthesized they are responsible for the replication of the plus strand (42S) genome into full-length minus strands. These molecules then serve as templates for the production of new 42S genomic RNAs. They also serve as templates for the synthesis of subgenomic (26S) RNA. This 4073 nucleotides long RNA is colinear with the last one-third of the genome, and its synthesis is internally initiated at the 26S promoter on the 42S minus strands (17, 18).

The capsid and envelope proteins are synthesized in different compartments, and they follow separate pathways through the cytoplasm, viz. the envelope proteins

are synthesized by membrane-bound ribosomes attached to the rough endoplasmic reticulum, and the capsid protein is synthesized by free ribosomes in the cytosol. However, the 26S RNA codes for all the structural proteins of the virus, and these are synthesized as a polyprotein precursor in the order C-E3-E2-6K-E1 (19). Once the capsid (C) protein has been synthesized it folds to act as a protease cleaving itself off the nascent chain (20, 21). The synthesized C proteins bind to the recently replicated genomic RNA to form new nucleocapsid structures in the cell cytoplasm.

The said cleavage reveals an N-terminal signal sequence in the nascent chain which is recognized by the signal recognition particle targeting the nascent chain - ribosome complex to the endoplasmic reticulum (ER) membrane (22, 23), where it is cotranslationally translocated and cleaved by signal peptidase to the three structural membrane proteins p62 (precursor form of E3/E2), 6K and E1 (24, 25). The translocational signals used during the synthesis of the structural proteins are illustrated in Fig. 2. The membrane proteins undergo extensive posttranslational modifications within the biosynthetic transport pathway of the cell. The p62 protein forms a heterodimer with E1 via its E3 domain in the endoplasmic reticulum (26). This dimer is transported out to the plasma membrane, where virus budding occurs through spike nucleocapsid interactions. At a very late (post-Golgi) stage of transport the p62 protein is cleaved to E3 and E2 (27), the forms that are found in mature virions. This cleavage activates the host cell binding function of the virion as well as the membrane fusion potential of E1. The latter activity is expressed by a second, low-pH activation step after the virus enters the endosomes of a new host cell and is responsible for the release of the viral nucleocapsid into the cell cytoplasm (28-32). The mature virus particles contain one single copy of the RNA

genome encapsidated within 180 copies of the capsid protein in a T=3 symmetry, and is surrounded by a lipid bilayer carrying 240 copies of the spike trimer protein consisting of E1+E2+E3 arranged in groups of three in a T=4 symmetry (33).

The SFV entry functions are activated and regulated by p62 cleavage and pH. More specifically, the p62-E1 heterodimers formed in the ER are acid resistant. When these heterodimers are transported to the plasma membrane via the Golgi complex the E1 fusogen cannot be activated in spite of the mildly acidic environment, since activation requires dissociation of the complex. As is illustrated in Fig. 1, the released virus particles contain E2E1 complexes. Since the association between E2 and E1 is sensitive to acidic pH, during entry of the virus into a host cell through endocytosis the acidic milieu of the endosome triggers the dissociation of the spike complex (E1 E2 E3) resulting in free E1. The latter can be activated for the catalysis of the fusion process between the viral and endosomal membranes in the infection process as disclosed above.

As indicated in the preceding parts of the disclosure, the alphavirus system, and especially the SFV system, has several unique features which are to advantage in DNA expression systems. These are summarized below with reference to Fig. 3.

1. Genome of positive polarity. The SFV RNA genome is of positive polarity, i.e. it functions directly as mRNA, and infectious RNA molecules can thus be obtained by transcription from a full-length cDNA copy of the genome.

2. Efficient replication. The infecting RNA molecule codes for its own RNA replicase, which in turn drives an efficient RNA replication. Indeed, SFV is one of the most efficiently replicating viruses known. Within a few hours up to 200.000 copies of the plus-RNAs are made in a single cell. Because of the abundance of

these molecules practically all ribosomes of the infected cell will be enrolled in the synthesis of the virus encoded proteins, thus overtaking host protein synthesis (34), and pulse-labelling of infected cells results in almost exclusive labelling of viral proteins. During a normal infection 10^5 new virus particles are produced from one single cell, which calculates to at least 10^8 protein molecules encoded by the viral genome (5).

3. Cytoplasmic replication. SFV replication occurs in the cell cytoplasm, where the virus replicase transcribes and caps the subgenomes for production of the structural proteins (19). It would obviously be very valuable to include this feature in a cDNA expression system to eliminate the many problems that are encountered in the conventional "nuclear" DNA expression systems, such as mRNA splicing, limitations in transcription factors, problems with capping efficiency and mRNA transport.

4. Late onset of cytopathic effects. The cytopathic effects in the infected cells appear rather late during infection. Thus, there is an extensive time window from about 4 hours after infection to up to 24 hours after infection during which a very high expression level of the structural proteins is combined with negligible morphological change.

5. Broad host range. This phenomenon is probably a consequence of the normal life cycle which includes transmission through arthropod vectors to wild rodents and birds in nature. Under laboratory conditions, SFV infects cultured mammalian, avian, reptilian and insect cells (35) (Xiong, et al, loc. cit.)

6. In nature SFV is of very low pathogenicity for humans. In addition, the stock virus produced in tissue culture cells is apparently apathogenic. By means of specific mutations it is possible to create conditionally lethal mutations of SFV, a feature that is of

great use to uphold safety when massproduction of virus stocks is necessary.

In the nucleotide and amino acid sequences the following abbreviations have been used in this specification:

Ala, alanine; Ile, isoleucine; leu, leucine; Met, methionine; Phe, phenylalanine; Pro, proline; Trp, tryptophan; Val, valine; Asn, asparagine; Cys, cysteine; Gln, glutamine; Gly, glycine; Ser, serine; Thr, threonine; Tys, tyrosine; Arg, arginine; His, histidine; Lys, lysine; Asp, aspartic acid; Glu, glutamic acid; A, adenine; C, cytosine; G, guanine; T, thymine; U, uracil.

The materials and the general methodology used in the following examples are disclosed below.

1. Materials. Most restriction enzymes, DNA Polymerase I, Klenow fragment, calf intestinal phosphatase, T4 DNA ligase and T4 Polynucleotide kinase were from Boehringer (Mannheim, FRG). SphI, StuI and KpnI together with RNase inhibitor (RNasin) and SP6 Polymerase were from Promega Biotec (Madison, WI). Sequenase (Modified T7 polymerase) was from United States Biochemical (Cleveland, Ohio). Proteinase K was from Merck (Darmstadt, FRG). Ribonucleotides, deoxyribonucleotides, dideoxyribonucleotides and the cap analogue $m^7G(5')ppp(5')G$ were from Pharmacia (Sweden). Oligonucleotides were produced using an Applied Biosystems synthesizer 380B followed by HPLC and NAP-5 (Pharmacia) purification. Spermidine, phenylmethylsulfonyl fluoride (PMSF), diethylpyrocarbonate (DEPC), bovine serum albumin (BSA), creatine phosphate and creatine phosphokinase were from Sigma (St. Louis, Mo). Pansorbin was from CalBiochem (La Jolla, CA). Agarose was purchased from FMC BioProducts (Rockland, Maine), and acrylamide from BioRad (Richmond, CA). L-[³⁵S]-methionine and α -[³⁵S]-dATP- α -S were from Amersham.

2. Virus growth and purification: BHK-21 cells were

grown in BHK medium (Gibco Life Technologies, Inc., New York) supplemented with 5 % fetal calf serum, 10 % tryptose phosphate broth, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and 2 mM glutamine. 90 % confluent monolayers were washed once with PBS and infected with SFV in MEM containing 0.2 % bovine serum albumin (BSA), 10 mM HEPES and 2 mM glutamine at a multiplicity of 0.1. Twenty-four hours post infection (p.i.) the medium was collected and cell debris removed by centrifugation at 8,000 xg for 20 min at 4°C. The virus was pelleted from the medium by centrifugation at 26,000 rpm for 1.5 h in an SW28 rotor at 4°C. The virus was resuspended in TN containing 0.5 mM EDTA.

3. Metabolic labeling and immunoprecipitation. Confluent monolayers of BHK cells grown in MEM supplemented with 10 mM HEPES, 2 mM glutamine, 0.2 % BSA, 100 IU/mol of penicillin and 100 µg/ml streptomycin, were infected at a multiplicity of 50 at 37°C. After 1 h p.i. the medium was replaced with fresh and growth continued for 3.5 h. The medium was removed and cells washed once with PBS and overlayed with methionine-free MEM containing 10 mM HEPES and 2 mM glutamine. After 30 min at 37°C the medium was replaced with the same containing 100 µCi/ml of [³⁵S]methionine (Amersham) and the plates incubated for 10 min at 37°C. The cells were washed twice with labeling medium containing 10X excess methionine and then incubated in same medium for various times. The plates were put on ice, cells washed once with ice-cold PBS and finally lysis buffer (1 % NP-40 - 50 mM Tris-HCl, pH 7.6 - 150 mM NaCl - 2 mM EDTA) containing 10 µg/ml PMSF (phenylmethylsulfonyl fluoride) was added. Cells were scraped off the plates, and nuclei removed by centrifugation at 6,000 rpm for 5 min at 4°C in an Eppendorf centrifuge. Immunoprecipitations of proteins was performed as described (31). Briefly, antibody was added to lysate and the mixture

kept on ice for 30 min. Complexes were recovered by binding to Pansorbin for 30 min on ice. Complexes were washed once with low salt buffer, once with high salt buffer, and once with 10 mM Tris-HCl, pH 7.5, before heating with gel loading buffer. To precipitate dhfr, SDS was added to 0.1 % and the mixture heated to 95°C for 2 min followed by addition of 10 volumes of lysis buffer. Anti-E1 [8.139], anti-E2 [5.1] (36), and anti-C [12/2] (37) monoclonals have been described. The human transferrin receptor was precipitated with the monoclonal antibody OKT-9 in ascites fluid. This preparation was provided by Thomas Ebel at our laboratory using a corresponding hybridoma cell line obtained from ATCC (American Typ Culture Collection) No CRL 8021.

15 Polyclonal rabbit anti-mouse dhfr was a kind gift from E. Hurt (European Molecular Biology Laboratory, Heidelberg, FRG) and rabbit anti-lysozyme has been described (38).

20 4. Immunofluorescence. To perform indirect immunofluorescence, infected cell monolayers on glass coverslips were rinsed twice with phosphate-buffered saline (PBS) and fixed in -20°C methanol for 6 min. After fixation, the methanol was removed and the coverslip washed 3 times with PBS. Unspecific antibody binding was blocked by incubation at room temperature with PBS containing 0.5 % gelatin and 0.25 % BSA. The blocking buffer was removed and replaced with same buffer containing primary antibody. After 30 min at room temperature the reaction was stopped by washing 3 times with PBS. Binding of secondary antibody (FITC-conjugated sheep anti-mouse [BioSys, Compiègne, France]) was done as for the primary antibody. After 3 washes with PBS and one rinse with water the coverslip was allowed to dry before mounting in Moviol 4-88 (Hoechst, Frankfurt am Main, FRG) containing 2.5 % DABCO (1,4-diazobicyclo-[2.2.2]-octane).

25 30 35 5. DNA procedures. Plasmids were grown in Escherichia

coli DH5 α (Bethesda Research Laboratories) [recA endA1 gyrA96 thiI hsdR17 supE44 relA1 Δ (lacZYA-argF)U169 ϕ 80dlacZ Δ (M15)]. All basic DNA procedures were done essentially as described (39). DNA fragments were isolated from agarose gels by the freeze-thaw method (40) including 3 volumes of phenol during the freezing step to increase yield and purity. Fragments were purified by benzoyl-naphthoyl-DEAE (BND) cellulose (Serva Feinbiochemica, Heidelberg, FRG) chromatography (41).

Plasmids used for production of infectious RNA were purified by sedimentation through 1 M NaCl followed by banding in CsCl (39). In some cases plasmids were purified by Qiagen chromatography (Diagen GmbH, Düsseldorf, FRG).

6. Site-directed oligonucleotide mutagenesis. For oligonucleotide mutagenesis, relevant fragments of the SFV cDNA clone were subcloned into M13mp18 or mp 19 (42) and transformed (43) into DH5 α FIQ [endA1 hsdR1 supE44 thiI recA1 gyrA96 relA1 ϕ 80dlac Δ (M15) Δ (lacZYA-argF)U169/F'proAB lacI q lacZ Δ (M15) Tn 5] (Bethesda Research Laboratories). RF DNA from these constructs was transformed into RZ1032 (44) [Hfr KL16 dutI ungI thiI relA1 supE44 zbd279:Tn10.], and virus grown in the presence of uridine to incorporate uracil residues into the viral genome. Single stranded DNA was isolated by phenol extraction from PEG precipitated phage. Oligonucleotides were synthesized on an Applied Biosystems 380B synthesizer and purified by gel filtration over NAP-5 columns (Pharmacia). The oligonucleotides

5'-CGGCCAGTGAATTCTGATTGGATCCGGGTAAATTGAATTACATCCC-TACGC
AAACG, 5'-GCGCACTATTATAGCACCCGGCTCCGGTAATTAAATT-GACGC
AAACGTTTACGGCCGCCGG and 5'-GCGCACTATTATAGCACCATG-GATCC
GGGTAAATTAAATTGACGTTTACGGCCGCCGGTGGCG were used to insert the new linker sites [BamHI-SmaI-XbaI] into the SFV cDNA clone. The oligonucleotides 5'-CGGCGGTCTTA-GATTGGTGCG and 5'-CGCGGGCGCCACCGGCGGCCG were used as sequencing primers (SP1 and SP2) up- and downstream of

the polylinker site. Phosphorylated oligonucleotides were used in mutagenesis with Sequenase (United States Biochemicals, Cleveland, Ohio) as described earlier (44, 45). In vitro made RF forms were transformed into DH5 α F'IQ and the resulting phage isolates analyzed for the presence of correct mutations by dideoxy sequencing according to the USB protocol for using Sequenase. Finally, mutant fragments were reinserted into the full-length SFV cDNA clone. Again, the presence of the appropriate mutations was verified by sequencing from the plasmid DNA. Deletion of the 6K region has been described elsewhere.

7. In vitro transcription. SpeI linearized plasmid DNA was used as template for in vitro transcription. RNA was synthesized at 37°C for 1 h in 10-50 μ l reactions containing 40 mM Tris-HCl (pH 7.6), 6 mM spermidine-HCl, 5 mM dithiothreitol (DTT), 100 μ g/ml of nuclease free BSA, 1 mM each of ATP, CTP and UTP, 500 μ M of GTP, 1 unit/ μ l of RNasin and 100-500 units/ml of SP6 RNA polymerase. For production of capped transcripts (46), the analogs $m^7G(5')ppp(5')G$ or $m^7G(5')ppp(5')A$ were included in the reaction at 1 mM. For quantitation of RNA production, trace amounts of [α -³²P]-UTP (Amersham) was included in the reactions and incorporation measured from trichloroacetic acid precipitates. When required, DNA or RNA was digested at 37°C for 10 min by adding DNase I or RNase A at 10 units/ μ g template or 20 μ g/ml respectively.

8. RNA transfection. Transfection of BHK monolayer cells by the DEAE-Dextran method was done as described previously (47). For transfection by electroporation, RNA was added either directly from the in vitro transcription reaction or diluted with transcription buffer containing 5 mM DTT and 1 unit/ μ l of RNasin. Cells were trypsinized, washed once with complete BHK-cell medium and once with ice-cold PBS (without MgCl₂ and CaCl₂) and finally resuspended in PBS to give 10⁷ cells/ml. Cells

were either used directly or stored (in BHK medium) on ice over night. For electroporation, 0.5 ml of cells were transferred to a 0.2 cm cuvette (BioRad), 10-50 μ l of RNA solution added and the solution mixed by inverting the cuvette. Electroporation was performed at room temperature by two consecutive pulses at 1.5 kV/25 μ F using a BioRad Gene Pulser apparatus with its pulse controller unit set at maximum resistance. After incubation for 10 min, the cells were diluted 1:20 in complete BHK-cell medium and transferred onto tissue culture plates. For plaque assays, the electroporated cells were plated together with about 3×10^5 fresh cells per ml and incubated at 37°C for 2 h, then overlayed with 1.8 % low melting point agarose in complete BHK-cell medium. After incubation at 37°C for 48 h, plaques were visualized by staining with neutral red.

9. Gel electrophoresis. Samples for sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) were prepared and run on 12 % separating gels with a 5 % stacking gel as previously described (48). For resolving the 6K peptide, a 10 % - 20 % linear acrylamide gradient gel was used. Gels were fixed in 10 % acetic acid - 30 % methanol for 30 min before exposing to Kodak XAR-5 film. When a gel was prepared for fluorography (49), it was washed after fixation for 30 min in 30 % methanol and then soaked in 1M sodium salicylate - 30 % methanol for 30 min before drying. Nucleic acids were run on agarose gels using 50 mM Tris-borate - 2.5 mM Na₂EDTA as buffer. For staining 0.2 μ g/ml of ethidium bromide was included in the buffer and gel during the run.

Example 1

In this example a full-length SFV cDNA clone is prepared and placed in a plasmid containing the SP6 RNA polymerase promoter to allow in vitro transcription of full-length and infectious transcripts. This plasmid which is designated pSP6-SFV4 has been deposited on 28

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European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wiltshire, U.K., and given the provisional accession number 91112826.

As illustrated in Fig. 4A-C the strategy for construction the SFV clone was to prime cDNA synthesis on several positions along the template RNA downstream of suitable restriction endonuclease sites defined by the known nucleotide sequence of the SFV RNA molecule.

Virus RNA was isolated by phenol-chloroform extraction from purified virus (obtainable among others from the Arbovirus collection in Yale University, New Haven, USA) and used as template for cDNA synthesis as previously described (50). First strand synthesis was primed at three positions, using 5'-TTTCTCGTAGTTCTCCTC-GTC as primer-1 (SFV coordinate 2042-2062) and 5'-GTTATCCCAGTGGTTGTTCTCGTAATA as primer-2 (SFV coordinate 3323-3349) and an oligo-dT₁₂₋₁₈ as primer -3 (3' end of SFV) Fig. 4A).

Second strand synthesis was preceded by hybridization of the oligonucleotide 5'-ATGGCGGATGTGTGACATACACGACGCC (identical to the 28 first bases of the genome sequence of SFV) to the first strand cDNA. After completion of second strand synthesis cDNA was trimmed and in all cases except in the case of the primer-1 reaction, the double-stranded adaptor 5'-AATTCAAAGCTTGCGGCCGCACTAGT / GTTCGAACGCCGGCGTGATCA-3' (5'-sticky-EcoRI-HindIII-NotI-XbaIII-SpeI-blunt-3') was added and the cDNA cloned into EcoRI cleaved pTZ18R (Pharmacia, Sweden) as described (51). The cloning of the 5' end region was done in a different way. Since SFV contains a HindIII site at position 1947, cDNA primed with primer-1 should contain this area and therefore HindIII could be used to define the 3' end of that cDNA. To obtain a restriction site at the very 5' end of the SFV, cDNA was cloned into SmaI-HindIII cut pGEM1 (Promega Biotech.,

Madison, WI). Since the SFV genome starts with the sequence 5'-ATGG, ligation of this onto the blunt CCC-3' end of the SmaI site created an NcoI site C'CATGG. Although the SFV sequence contains 3 NcoI sites, none of these are within the region preceding the HindIII site, and thus these 5' end clones could be further subcloned as NcoI-HindIII fragments into a vector especially designed for this purpose (see below). The original cDNA clones in pGEM1 were screened by restriction analysis and all containing inserts bigger than 1500 bp were selected for further characterization by sequencing directly from the plasmid into both ends of the insert, using SP6 or T7 sequencing primers. The SFV 5'-end clones in pTZ18R were sequenced using lac sequencing primers. To drive in vitro synthesis of SFV RNA the SP6 promoter was used. Cloning of the SFV 5' end in front of this promoter without adding too many foreign nucleotides required that a derivative of pGEM1 had to be constructed. Hence, pGEM1 was opened at EcoRI and Bal31 deletions were created, the DNA blunted with T4 DNA polymerase and an NcoI oligonucleotide (5'-GCCATGGC) added. The clones obtained were screened by colony hybridization (39) with the oligonucleotide 5'-GGTGACACTATAGCCATGGC designed to pick up (at suitable stringency) the variants that had the NcoI sequence immediately at the transcription initiation site of the SP6 promoter (G underlined). Since the Bal31 deletion had removed all restriction sites of the multicloning site of the original plasmid, these were restored by cloning a PvuI-NcoI fragment from the new variant into another variant of pGEM1 (pDH101) that had an NcoI site inserted at its HindIII position in the polylinker. This created the plasmid pDH201. Finally, the adaptor used for cloning the SFV cDNA was inserted into pDH201 between the EcoRI and PvuII sites to create plasmid pPLH211 (Fig. 4B). This plasmid was then used as recipient for SFV cDNA fragments in the assembly of the

full-length clone by combining independent overlapping subclones using these sites. The fragments and the relevant restriction sites used to assemble the full-length clone, pSP6-SFV4, are depicted in (Fig. 4A). For the 5'-end, the selected fragment contained the proper SFV sequence 5'-ATGG, with one additional G-residue in front. When this G-residue was removed it reduced transcription efficiency from SP6 but did not affect infectivity of the in vitro made RNA. Thus, the clone used for all subsequent work contains the G-residue at the 5' end. For the 3'-end of the clone, a cDNA fragment containing 69 A-residues was selected. By inclusion of the unique SpeI site at the 3'-end of the cDNA, the plasmid can be linearized to allow for runoff transcription in vitro giving RNA-carrying 70 A-residues. Fig. 4C shows the 5' and 3' border sequences of the SFV cDNA clone. The general outline how to obtain and demonstrate infectivity of the full-length SFV RNA is depicted in Fig. 6. The complete nucleotide sequence of the pSP6-SFV4 SP6 transcript together with the amino acid sequences of the nonstructural and the structural polyproteins is shown in Fig. 5.

Typically, about 5 µg of RNA per 100 ng of template was obtained using 10 units of polymerase, but the yield could be increased considerably by the use of more enzyme. The conditions slightly differ from those reported earlier for the production of infectious transcripts of alphaviruses (52) (47). A maximum production of RNA was obtained with rNTP concentrations at 1 mM. However, since infectivity also is dependent on the presence of a 5'cap structure optimal infectivity was obtained when the GTP concentration in the transcription reaction was halved. This drop had only a marginal effect on the amounts of RNA produced but raised the specific infectivity by a factor of 3 (data not shown).

The cDNA sequence shown in Fig. 5 has been used in the following examples. However, sequences having one

or a few nucleotides, which differ from those shown in Fig. 5, could also be useful as vectors, even if these might be less efficient as illustrated above with the SFV cDNA sequence lacking the first 5'-G nucleotide in Fig. 5.

Example 2.

In this example the construction of SFV DNA expression vectors is disclosed.

The cDNA clone coding for the complete genome of SFV obtained in Example 1 was used to construct a SFV DNA expression vector by deletion of the coding region of the 26S structural genes to make way for heterologous inserts. However, the nonstructural coding region, which is required for the production of the nsP1-4 replicase complex is preserved. RNA replication is dependent on short 5' (nt 1-247) (53, 54, 55) and 3' (nt 11423-11441) sequence elements (56, 57), and therefore, also these had to be included in the vector construct, as had the 26S promoter just upstream of the C gene (17, 18).

As is shown in Fig. 7, first, the XbaI (6640)-NsiI (8927) fragment from the SFV cDNA clone pSP6-SFV4 from Example 1 was cloned into pGEM7zf(+) (Promega Corp., WI, USA) (Step A). From the resulting plasmid, pGEM7zf(+) - SFV, the EcoRI fragment (SFV coordinates 7391 and 88746) was cloned into M13mp19 to insert a BamHI - XbaI - SmaI polylinker sequence immediately downstream from the 26S promoter site using site-directed mutagenesis (step B). Once the correct mutants had been verified by sequencing from M13 ssDNA (single stranded), the EcoRI fragments were reinserted into pGEM7zf(+) - SFV (step C) and then cloned back as XbaI-NsiI fragments into pSP6-SFV4 (step D). To delete the major part of the cDNA region coding for the structural proteins of SFV, these plasmids were then cut with AsuII (7783) and NdeI (11033), blunted using Klenow fragment in the presence of all four nucleotides, and religated to create the

5 final vectors designated pSFV1, pSFV2 and pSFV3, respectively (step E). The vectors retain the promoter region of the 26S subgenomic RNA and the last 49 amino acids of the E1 protein as well as the complete non-coding 3' end of the SFV genome.

10 In the vectors the subgenomic (26S) protein coding portion has been replaced with a polylinker sequence allowing the insertional cloning of foreign cDNA sequences under the 26S promoter. As is shown in Fig. 8 these three vectors have the same basic cassette inserted downstream from the 26S promoter, i.e. a polylinker (BamHI-SmaI-XbaI) followed by a translational stop-codons in all three reading frames. The vectors differ as to the position where the polylinker cassette has been inserted. In pSFV1 the cassette is situated 31 bases downstream of the 26S transcription initiation site. The initiation motive of the capsid gene translation is identical to the consensus sequence (58).

15 Therefore, this motive has been provided for in pSFV2, where it is placed immediately after the motive of the capsid gene. Finally, pSFV3 has the cassette placed immediately after the initiation codon (AUG) of the capsid gene. Sequencing primers (SP) needed for checking both ends of an insert have been designed to hybridize either to the 26S promoter region (SP1), or to the region following the stop codon cassette (SP2).

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30 Note that the 26S promoter overlaps with the 3'-end of the nsP4 coding region. For pSFV2, the cloning site is positioned immediately after the translation initiation site of the SFV capsid gene. For pSFV3, the cloning site is positioned three nucleotides further downstream, i.e. immediately following to the initial AUG codon of the SFV capsid gene. The three translation stop codons following the polylinker are boxed. The downstream sequencing primer (SP1) overlaps with the 26S promoter, and the upstream sequencing primer (SP2)

35

overlaps the XmaIII site.

Example 3

In this example an in vivo packaging system encompassing helper virus vector constructs is prepared.

The system allows SFV variants defective in structural protein functions, or recombinant RNAs derived from the expression vector construct obtained in Example 2, to be packaged into infectious virus particles. Thus, this system allows recombinant RNAs to be introduced into cells by normal infection. The helper vector, called pSFV-Helper1, is constructed by deleting the region between the restriction endonuclease sites AccI (308) and AccI (6399) of pSP6-SFV4 obtained in Example 1 by cutting and religation as shown in Fig. 7, step F. The vector retains the 5' and 3' signals needed for RNA replication. Since almost the complete nsP region of the Helper vector is deleted, RNA produced from this construct will not replicate in the cell due to the lack of a functional replicase complex. As is shown in Fig. 9, after transcription in vitro of pSFV1-recombinant and helper cDNAs, helper RNA is cotransfected with the pSFV1 - recombinant derivative, the helper construct providing the structural proteins needed to assemble new virus particles, and the recombinant providing the nonstructural proteins needed for RNA replication, SFV particles comprising recombinant genomes being produced. The cotransfection is preferably produced by electroporation as is disclosed in Example 6 and preferably BHK cells are used as host cells.

To package the RNA a region at the end of nsP1 is required, an area which has been shown to bind capsid protein (57, 59). Since the Helper lacks this region, RNA derived from this vector will not be packaged and hence, transfections with recombinant and Helper produces only virus particles that carry recombinant-derived RNA. It follows that these viruses cannot be

passaged further and thus provide a one-step virus stock. The advantage is that infections with these particles will not produce any viral proteins.

Example 4

5 This example illustrates the construction of variants of the full-length SFV cDNA clone from Example 1 that allow insertion of foreign DNA sequences encoding foreign epitopes, and the production of recombinant (chimaeric) virus carrying said foreign epitopes as 10 integral parts of the p62, E2 or E1 spike proteins.

To this end, a thorough knowledge of the function, topology and antigenic structure of the E2 and E1 envelope proteins has been of the essence. Earlier studies on the pathogenicity of alphaviruses have shown 15 that antibodies against E2 are type-specific and have good neutralizing activity while those against E1 generally are group-specific and are nonneutralizing (5). However, not until recently have antigenic sites of the closely related alphaviruses SFV, Sindbis, and Ross River been mapped and correlated to the level of 20 amino acid sequence (60, 61, 62, 63). These studies have shown that the most dominant sites in question are at amino acid positions 216, 234 and 246-251 of the SFV 25 E2 spike protein. Interestingly, these three sites are exactly the same as the ones predicted by computer analysis. In the present example domain 246-251 was used, since this area has a highly conserved structure and hydropathy profile within the group of alpha-viruses. Insertion of a gene encoding a foreign epitope 30 into the 246-251 region of the pSP6-SFV4 p62 protein yields particles with one new epitope on each heterodimer, i.e. 240 copies.

To create a unique restriction endonuclease site that 35 would allow specific insertion of foreign epitopes into the E2 portion of the SFV genome, a BamHI site was inserted by site directed mutagenesis using the oligonucleotide 5'-GATCGGCCTAGGAGCCGAGAGCCC.

Example 5

In this example a conditionally lethal variant of SFV is constructed from the SFV cDNA obtained in Example 1, which variant carries a mutation in the p62 protein resulting in a noncleavable form of said protein, with the result that this variant as such cannot infect new host cells, unless first cleaved with exogenously added protease.

As illustrated in Fig. 10, this construct can be advantageously used as a vaccine carrier for foreign epitopes, since this form of the virus cannot enter new host cells although assembled with wild type efficiency in transfected cells. The block can be overcome by trypsin treatment of inactive virus particles. This converts the particle into a fully entry-competent form which can be used for amplification of this virus variant stock.

Once activated the SFV variant will enter cells normally through the endocytic pathway and start infection. Viral proteins will be made and budding takes place at the plasma membrane. However, all virus particles produced will be of inactive form and the infection will thus cease after one round of replication. The reason for the block in infection proficiency is a mutation which has been introduced by site directed mutagenesis into the cleavage site of p62. This arginine to leucine substitution (at amino acid position 66 of the E3 portion of the p62 protein) changes the consensus features of the cleavage site so that it will not be recognized by the host cell proteinase that normally cleaves the p62 protein to the E2 and E3 polypeptides during transport to the cell surface. Instead, only exogenously added trypsin will be able to perform this cleavage, which in this case occurs at the arginine residue 65 immediately preceding the original cleavage site. As this cleavage regulates the activation of the entry function potential of the

virus by controlling the binding of the entry spike subunit, the virus particle carrying only uncleaved p62 will be completely unable to enter new host cells.

5 The creation of the cleavage deficient mutation E2 has been described earlier (29). An AsuII - NsiI fragment spanning this region was then isolated and cloned into the full-length cDNA clone pSP6-SFV4.

Example 6

10 In this example transfection of BHK cells with SFV RNA molecules transcribed in vitro from full-length cDNA from Example 1 or variants thereof or the SFV vectors from Example 2, which comprise exogenous DNA, is disclosed. The transfection is carried out by electroporation which is shown to be very efficient at 15 optimized conditions.

15 BHK cells were transfected with the above SFV RNA molecules by electroporation and optimal conditions were determined by varying parameters like temperature, voltage, capacitance, and number of pulses. Optimal transfection was obtained by 2 consecutive pulses of 20 1.5 kV at 25 μ F, under which negligible amounts of cells were killed. It was found that it was better to keep the cells at room temperature than at 0°C during the whole procedure. Transfection by electroporation 25 was also measured as a function of input RNA. As expected, an increase in transfection frequency was not linearly dependent on RNA concentration, and about 2 μ g of cRNA were needed to obtain 100 % transfection.

30 On comparison with conventional transfection, this is a great improvement. For example, with DEAE-Dextran transfection optimally, only 0.2 % of the cells were transfected:

Example 7

35 This example illustrates heterologous gene expression driven by the SFV vector, pSFV1 from Example 2, for genes encoding the 21 kD cytoplasmic mouse dihydro-folate reductase (dhfr), the 90 kD membrane protein

human transferrin receptor (TR), and finally the 14 kD secretory protein chicken lysozyme. The dhfr gene was isolated from pGEM2-dhfr (64) as a BamHI-HindIII fragment blunted with Klenow fragment and inserted into SmaI-cut pSFV1. The transferrin receptor gene was first cloned from pGEM1-TR (64, 65) as an XbaI-EcoRI fragment into pGEM7ZF(+) and subsequently from there as a BamHI fragment into pSFV1. Finally, a BamHI fragment from pGEM2 carrying the lysozyme gene (21) was cloned into pSFV1.

To study the expression of the heterologous proteins, in vitro-made RNA of the dhfr and TR constructs was electroporated into BHK cells. RNA of wild type SFV was used as control. At different time points post electroporation (p.e.) cells were pulse-labeled for 10 min followed by a 10 min chase, whereafter the lysates were analyzed by gel electrophoresis and autoradiography. The results are shown in Figure 11. More specifically, BHK cells were transfected with RNAs of wild type SFV, pSFV1-dhfr, and pSFV1-TR, pulse-labeled at 3, 6, 9, 12, 15 and 24 h p.e. Equal amounts of lysate were run on a 12 % gel. The 9 h sample was also used in immunoprecipitation (IP) of the SFV, the dhfr and the transferrin receptor proteins. Cells transfected with pSFV1-lysozyme were pulse-labeled at 9 h p.e. and then chased for the times (hours) indicated. An equal portion of lysate or medium was loaded on the 13,5 % gel. IP represents immunoprecipitation from the 1 h chase lysate sample. The U-lane is lysate of labeled but untransfected cells. At 3 h p.e. hardly any exogenous proteins were made, since the incoming RNA starts with minus strand synthesis which does not peak until about 4-5 h p.e. (5). At this time point, almost all labeled proteins were of host origin. In contrast, at 6 h p.e. the exogenous proteins were synthesized with great efficiency, and severe inhibition of host protein synthesis was evident. This was even more striking at 9 h

p.e., when maximum shut down had been reached.

Efficient production of the heterologous proteins continued up to 24 h p.e., after which production slowed down (data not shown), indicating that the cells had entered a stationary phase.

Since chicken lysozyme is a secretory protein, its expression was analyzed both from cell lysates and from the growth medium. Cells were pulse-labeled at 9 h p.e. and then chased up to 8 h. The results are shown in Fig. 11. Although lysozyme was slowly secreted, almost all labeled material was secreted to the medium during the chase.

Example 8

This example illustrates the present in vivo packaging system.

In vitro-made RNA of pSFV1-TR was mixed with Helper RNA at different ratios and these mixtures were co-transfected into BHK cells. Cells were grown for 24 h after which the culture medium was collected and the virus particles pelleted by ultracentrifugation. The number of infectious units (i.u.) was determined by immunofluorescence. It was found that a 1:1 ratio of Helper and recombinant most efficiently produced infectious particles, and on the average 5×10^6 cells yielded 2.5×10^9 i.u. The infectivity of the virus stock was tested by infecting BHK cells at different multiplicities of infection (m.o.i.). In Fig. 11 the results for expression of human transferrin receptor in BHK cells after infection by such in vivo packaged particles carrying pSFV1-TR recombinant RNA is shown to the lower right. 200 μ l of virus diluted in MEM (including 0,5% BAS and 2 mM glutamine) was overlaid on cells to give m.o.i. values ranging from 5 to 0.005. After 1 h at 37°C, complete BHK medium was added and growth continued for 9 h, at which time a 10 min pulse (100 μ Ci 35 S-methionine/ml) and 10 min chase was performed, and the cells dissolved in lysis buffer. 10

μl out of the 300 μl lysate (corresponding to 30,000 cells) was run on the 10 % gel, and the dried gel was exposed for 2 h at -70°C. Due to the high expression level, only 3,000 cells are needed to obtain a distinct band on the autoradiograph with an over night exposure.

5 Thus, it was found that efficient protein production and concomitant host protein shut-off occurred at about 1 i.u. per cell. Since one SFV infected cell produces on the average 10^8 capsid protein molecules, it follows 10 that a virus stock produced from a single electroporation can be used to produce 10^{17} protein molecules equaling about 50 mg of protein.

15 From the foregoing experimental results it is obvious that the present invention is related to very useful and efficient expression system which lacks several of the disadvantages of the hitherto existing expression system. The major advantages of the present system are shortly summarized as follows:

- 20 (1) High titre recombinant virus stocks can be produced in one day by one transfection experiment. There is no need for selection/screening, plaque purification and amplification steps. This is appreciated since an easy production of recombinant virus is especially important in experiments where the phenotypes of large series of mutants have to be characterized.
- 25 (2) The recombinant virus stock is free from helper virus since only the recombinant genome but not the helper genome contains a packaging signal.
- 30 (3) The recombinant virus can be used to infect the recombinant genome in a "natural" and nonleaky way into a large variety of cells including insect and most higher eukaryotic cell types. Such a wide host range is very useful for an expression system.

especially when cell-type-specific posttranslational modification reactions are required for the activity of the expressed protein.

- (4) The level of protein expression obtained is extremely high, the level corresponding to those of the viral proteins during infection. There is also a host cell protein shut-off which makes it possible to follow the foreign proteins clearly in cell lysates without the need for antibody mediated antigen concentration. This will facilitate DNA expression experiments in cell biology considerably. Furthermore, problems of interference by the endogenous counter part to an expressed protein (i.e. homo-oligomerization reactions) can be avoided.

Example 9

This example illustrates epitope carriers.

A very important example where vaccine development is of the utmost importance concerns the acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus HIV-1 (66, 67). Sofar, all attempts to produce an efficient vaccine against HIV-1 have failed, although there was a very recent report that vaccination with disrupted SIV-1 (Simian immunodeficiency virus) to a certain extent may give protection against infections of that virus (68). However, development of safe and effective vaccine against HIV-1 will be very difficult due to the biological properties of the virus. In the present example one epitope of HIV-1 was inserted into an antigenic domain of the E2 protein of SFV. The epitope used is located in glycoprotein gp120 of HIV-1, spanning amino acids 309-325. This forms the variable loop of HIV-1 and is situated immediately after an N-glycosylated site.

A chimaera was constructed where the 309-325 epitope of HIV was inserted into the BamHI site using cassette

insertion of ready-made oligonucleotides encoding the HIV epitope. The required base substitutions at the BamHI site did not lead to any amino acid changes in the vector, although two amino acids (Asp and Glu) changed places. This change did not have any deleterious effect since in vitro made vector RNA induced cell infection with wild type efficiency. Fig. 12 shows the sequences in the area of interest in the epitope carrier. In preliminary experiments, it has been shown that chimaeric proteins were produced. The proteins can be immunoprecipitated with anti-HIV antibodies. It is to be expected that these are also used for production of chimaeric virus particles that can be used for vaccine preparation against HIV. Such particles are shown in Fig. 12, lower part.

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Claims

1. An RNA molecule derived from an alphavirus RNA genome and capable of efficient infection of animal host cells, which RNA molecule comprises the complete alphavirus RNA genome regions, which are essential to replication of the said alphavirus RNA, and further comprises an exogenous RNA sequence capable of expressing its function in said host cell, said exogenous RNA sequence being inserted into a region of the RNA molecule which is non-essential to replication thereof.

10 2. The RNA of claim 1, wherein the said alphavirus is Semliki Forest virus (SFV).

15 3. The RNA of claim 1 or 2, wherein the exogenous RNA sequence encodes a protein, a polypeptide or a peptide sequence defining an exogenous antigenic epitope or determinant.

4. The RNA of claim 3 wherein the exogenous RNA sequence encodes an epitope sequence of a structural protein of an immunodeficiency virus inclusive of the human immunodeficiency virus (HIV) types.

20 5. The RNA of any preceding claim, wherein the alphavirus derived RNA molecule regions comprise a 5' terminal portion, the coding region(s) for non structural proteins required for RNA replication, the subgenome promoter region and a 3' terminal portion of said viral RNA.

25 6. The RNA of claim 2, 3 or 5, wherein the exogenous RNA sequence encodes a foreign polypeptide or protein and is integrated into the SFV subgenomic 26S RNA substituting deleted parts thereof.

30 7. The RNA of claim 2, 3, 4 or 5, wherein the exogenous RNA sequence encodes a foreign viral epitopic peptide sequence and is located in a region of the RNA coding for structural alphavirus proteins enabling the exogenous RNA to be expressed as said viral epitope as part of the matured virus particle.

35 8. The RNA of claim 2, 3, 4 or 5, wherein the exogenous RNA sequence encodes a foreign viral epitopic peptide sequence inserted into the p62 spike precursor subunit encoding region of the SFV genome.

40 9. An RNA expression vector comprising the RNA of any preceding claim packaged into infectious particles comprising the RNA within the alphavirus nucleocapsid and surrounded by membrane with alphavirus spike proteins.

10. The vector of claim 9, wherein the RNA has a total size corresponding to the wild type alphavirus RNA genome or deviating therefrom to an extent compatible with package of the RNA into the infectious particles.
- 5 11. DNA transcription vector comprising a cDNA having one strand complementary to the RNA of any of claims 1 to 8.
- 10 12. A DNA expression vector comprising a full-length or partial cDNA complementary to alphavirus RNA or parts thereof and located immediately downstream of the SP6 RNA polymerase promoter and having a 5'ATGG or 5'GATGG or any other 5' terminus and a TTTCCA₆₉ACTAGT or any other 3' terminus.
- 15 13. The vector of claim 12 having portions of the viral cDNA deleted, the deletions comprising the complete or part of the region(s) encoding the virus structural proteins, and further comprising an integrated polylinker region, which may correspond to BamHI-SmaI-XmaI, inserted at a location which enables an exogenous DNA fragment encoding a foreign polypeptide or protein to be inserted into the vector cDNA for subsequent expression in an animal host cell.
- 20 14. The vector of claim 12 or 13 wherein the alphavirus is SFV.
- 25 15. The vector of claim 12 or 14 comprising full-length cDNA and further comprising an exogenous DNA fragment encoding a foreign epitopic peptide sequence or antigenic determinant inserted into a region of the viral structural proteins.
16. The vector of claim 15 wherein the exogenous DNA fragment is inserted into the p62 spike precursor subunit encoding region of the SFV cDNA.
- 30 17. The vector of any preceding claim comprising an SFV derived cDNA which carries a conditionally lethal SFV mutation in the region encoding the p62 cleavage site, a cellularly uncleavable but extracellularly cleavable form of p62 being expressed.
- 35 18. The vector of claim 13 comprising SFV-derived cDNA, the vector being pSFV1, pSFV2 or pSFV3 having a structure as shown in Fig. 8.
19. An RNA transcript derived from transcription of the DNA-vector of any of claims 12-18 carrying an exogenous DNA fragment.
- 40 20. A method to produce the vector of claim 9 or 10.

wherein the alphavirus derived RNA lacks part of or the complete region(s) encoding the structural viral proteins, the method comprising cotransfection of animal host cells with the RNA transcript of claim 19, wherein the alphavirus RNA lacks part(s) of or the complete region(s) encoding the viral structural proteins, with helper RNA transcribed in vitro from a helper DNA vector and culturing the host cells.

5 21. The method of claim 20 wherein the cotransfection is produced by electroporation of the host cells.

10 22. Helper vector for use in the method according to claim 20 or 21, said vector being comprised of the DNA vector of claim 12 wherein the regions encoding non structural virus proteins are almost completely deleted, including sequences encoding RNA signals for packaging of RNA into nucleocapsid particles, but the 5' and 3' signals needed for RNA replication and the region encoding the promoter for the structural sub-genome are in addition to those encoding the structural region preserved.

20 23. Helper vector of claim 22 wherein the cDNA has its origin from SFV and the deletion extends from the AccI (308) to the AccI (6399) restriction endonuclease site of the full-length cDNA vector of claim 12.

25 24. Helper vector of claim 22 and 23 where the structural region contains the mutation described in claim 17 or another conditionally lethal mutation.

25 25. The method of claim 20 wherein cells transformed to produce helper RNA according to claims 20, 22 or 23 are transfected with RNA transcript of claim 19.

30 26. A host cell of animal origin transformed with the RNA of any of claims 1-8, the DNA transcription vector of claims 11 or the DNA vector of any of claims 12-18 carrying an exogenous DNA fragment.

35 27. The host cell of claim 26 wherein the cell is an avian, a mammalian, a reptilian, an amphibian, an insecticidal or a fish cell.

28. The host cell of claim 27 which is the hamster BHK cell.

40 29. A method to produce the transformed host cell of claim 26, 27 or 28 comprising transfection of the cell with the RNA of any of claim 1-8, with the cDNA of claim 11 or of any of

claims 12-18 carrying an exogenous DNA fragment or infection of the cell with the infectious viral particles of claim 9 or 10.

30. The method of claim 29 wherein the transfection is produced by electroporation of the host cell.

5 31. A method for the production of a polypeptide or protein comprising infection of animal host cells with infectious particles according to claim 9 or 10, containing exogenous RNA encoding said polypeptide or protein and produced according to method of claim 20 or 21, culturing the said transformed cells to express the exogenous RNA and isolation and purification of the product formed by said expression.

10 32. A method for the production of a polypeptide or protein comprising in vitro transcription of the cDNA of the vector of any of claims 11-18 carrying an exogenous DNA fragment coding for the polypeptide or protein, transfection of animal host cells with the produced RNA transcript, transformed animal host cells being obtained harbouring the RNA transcript, culturing the said transformed cells to express the exogenous RNA and isolation and purification of the product formed by said expression.

15 33. The method of claim 32 wherein the vector cDNA is comprised of the cDNA of the vector of claim 17 carrying the exogenous DNA fragment.

20 34. An antigen consisting of a chimaeric alphavirus having an exogenous epitopic peptide sequence or antigenic determinant inserted into its structural proteins.

25 35. The antigen of claim 34 wherein the chimaeric alphavirus is derived from SFV.

30 36. The antigen of claim 34 or 35, wherein the exogenous epitopic peptide sequence is comprised of an epitopic peptide sequence derived from a structural protein of a virus belonging to the immunodeficiency virus class inclusive of the human immunodeficiency virus types.

35 37. Vaccine preparation comprising the antigen of claim 34, 35 or 36 as immunizing component.

40 38. Vaccine of claim 37 wherein the chimaeric alphavirus is attenuated by comprising the conditionally lethal SFV mutation of claim 17, an amber (stop codon) a temperature sensitive mutation or other mutation in its genome.

39. A method for the production of an antigen of claim

34, 35 or 36 comprising

a) in vitro transcription of the cDNA of the vector of any of claims 11-18 carrying an exogenous DNA fragment encoding the foreign epitopic peptide sequence or antigenic determinant and transfection of animal host cells with the produced RNA transcript, or

b) transfection of animal host cells with the said cDNA of the above step a),

culturing the transfected cells and recovering the chimaeric alphavirus antigen.

40. The method of claim 32, 33 or 39 wherein the transfection is produced by electroporation of the host cell.

41. A method for the production of an antigen in an organism by using in vivo infection with infectious particles according to claim 9 or 10 containing exogenous RNA encoding an exogenous epitopic peptide sequence or antigenic determinant, and produced according the claim 20 or 21.

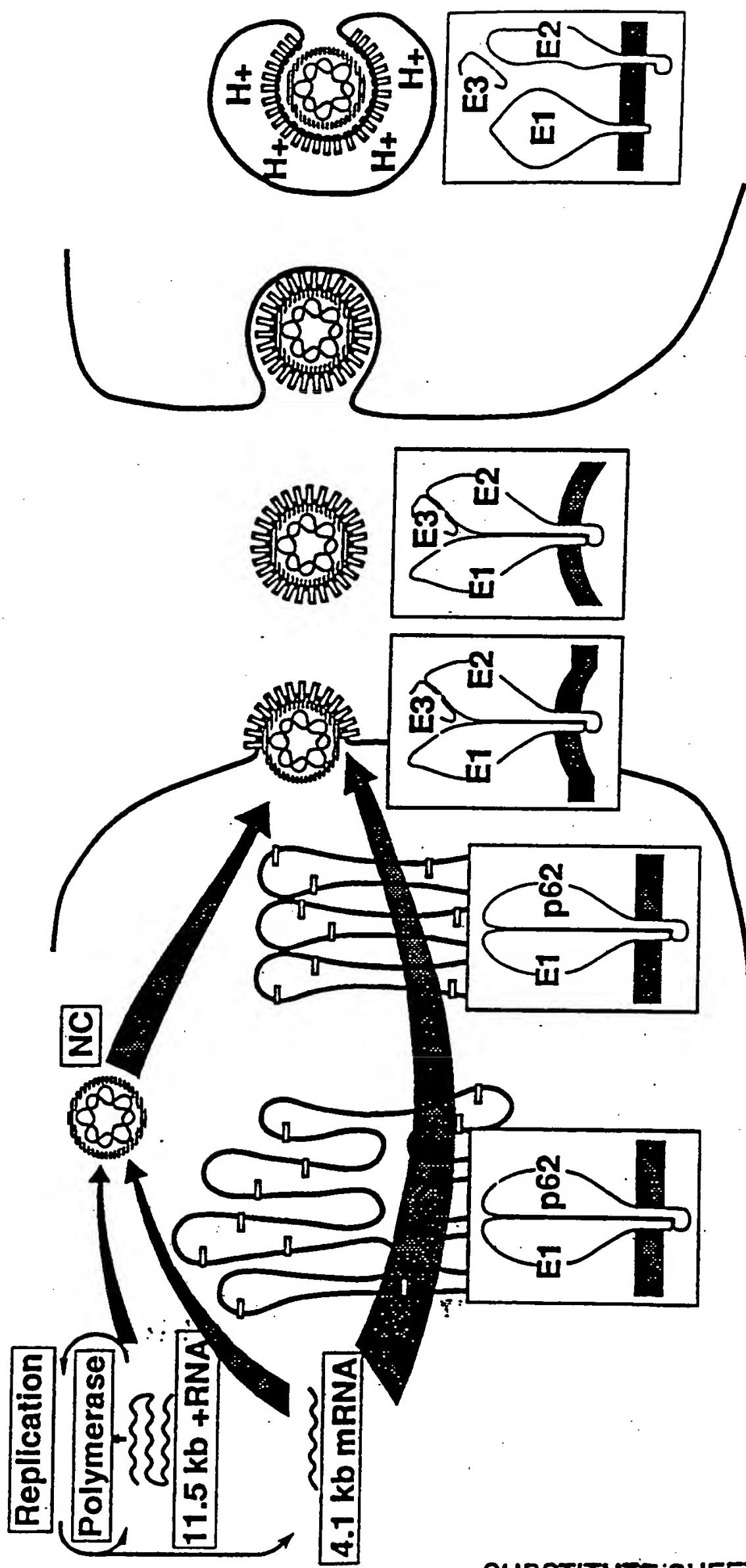
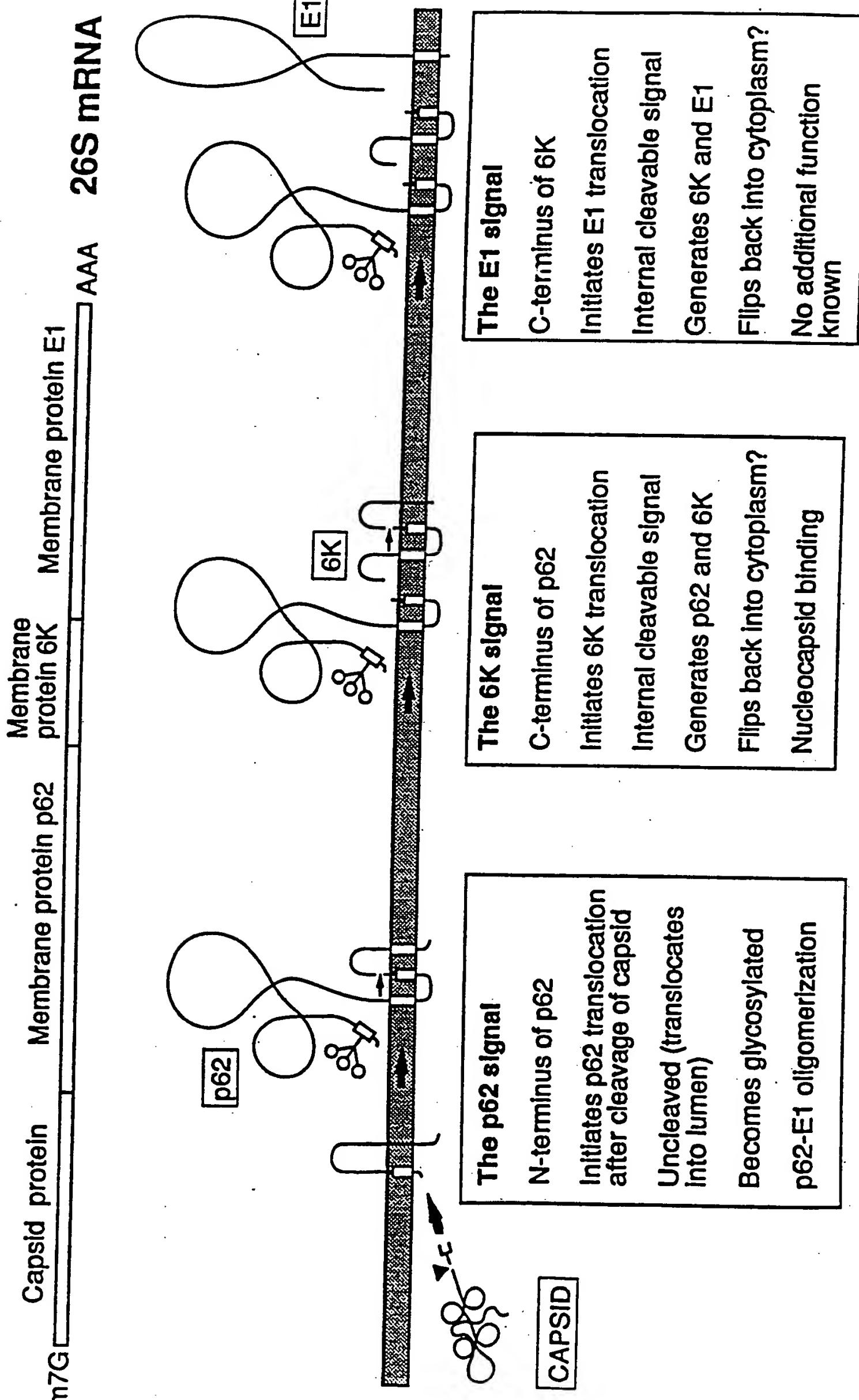
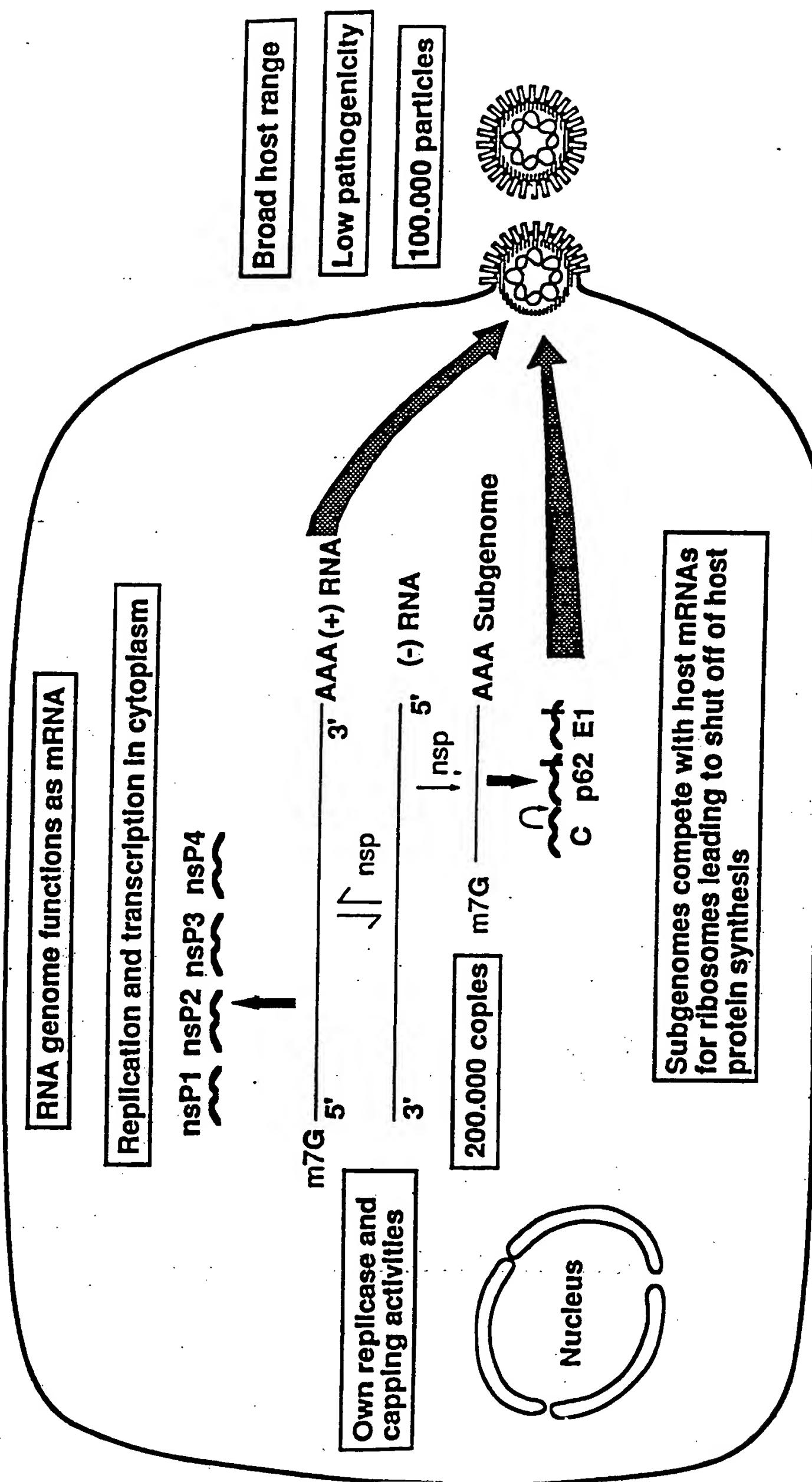


Figure 1

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**Figure 2**

**Figure 3**

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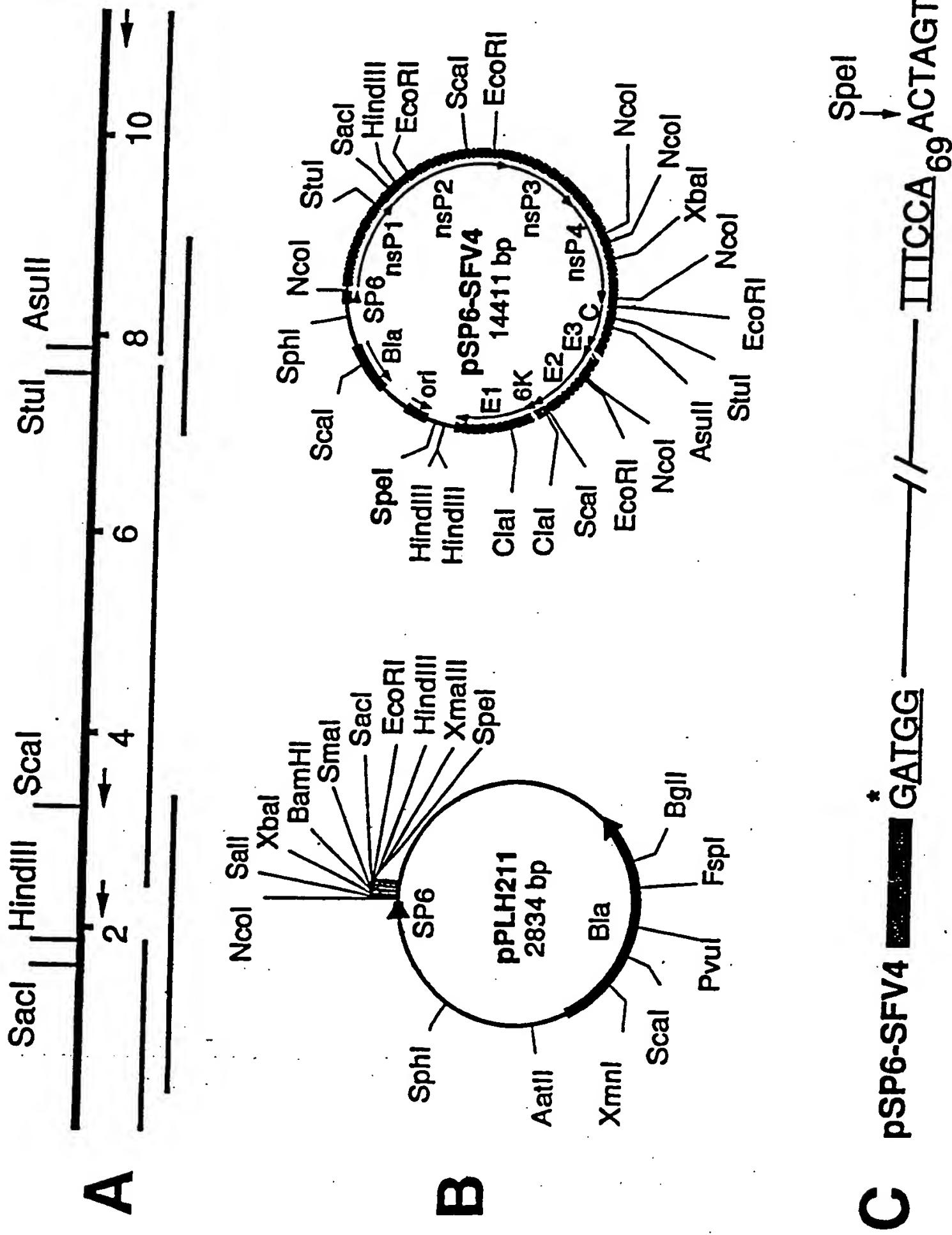


Figure 4

Figure 5 (1)

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GATGGCGGAT GTGTGACATA CACGACGCCA AAAGATTTTG TTCCAGCTCC TGCCACCTCC 60
 GCTACGCGAG AGATTAACCA CCCACG ATG GCC GCC AAA GTG CAT GTT GAT ATT 113
 Met Ala Ala Lys Val His Val Asp Ile
 5

GAG GCT GAC AGC CCA TTC ATC AAG TCT TTG CAG AAG GCA TTT CCG 158
 Glu Ala Asp Ser Pro Phe Ile Lys Ser Leu Gln Lys Ala Phe Pro
 10 15 20

TCG TTC GAG GTG GAG TCA TTG CAG GTC ACA CCA AAT GAC CAT GCA 203
 Ser Phe Glu Val Glu Ser Leu Gln Val Thr Pro Asn Asp His Ala
 25 30 35

AAT GCC AGA GCA TTT TCG CAC CTG GCT ACC AA. TTG ATC GAG CAG 248
 Asn Ala Arg Ala Phe Ser His Leu Ala Thr Lys Leu Ile Glu Gln
 40 45 50

GAG ACT GAC AAA GAC ACA CTC ATC TTG GAT ATC GGC AGT GCG CCT 293
 Glu Thr Asp Lys Asp Thr Leu Ile Leu Asp Ile Gly Ser Ala Pro
 55 60 65

TCC AGG AGA ATG ATG TCT ACG CAC AAA TAC CAC TGC GTA TGC CCT 338
 Ser Arg Arg Met Met Ser Thr His Lys Tyr His Cys Val Cys Pro
 70 75 80

ATG CGC AGC GCA GAA GAC CCC GAA AGG CTC GAT AGC TAC GCA AAG 383
 Met Arg Ser Ala Glu Asp Pro Glu Arg Leu Asp Ser Tyr Ala Lys
 85 90 95

AAA CTG GCA GCG GCC TCC GGG AAG GTG CTG GAT AGA GAG ATC GCA 428
 Lys Leu Ala Ala Ala Ser Gly Lys Val Leu Asp Arg Glu Ile Ala
 100 105 110

GGA AAA ATC ACC GAC CTG CAG ACC GTC ATG GCT ACG CCA GAC GCT 473
 Gly Lys Ile Thr Asp Leu Gln Thr Val Met Ala Thr Pro Asp Ala
 115 120 125

GAA TCT CCT ACC TTT TGC CTG CAT ACA GAC GTC ACG TGT CGT ACG 518
 Glu Ser Pro Thr Phe Cys Leu His Thr Asp Val Thr Cys Arg Thr
 130 135 140

GCA GCC GAA GTG GCC GTA TAC CAG GAC GTG TAT GCT GTA CAT GCA 563
 Ala Ala Glu Val Ala Val Tyr Gln Asp Val Tyr Ala Val His Ala
 145 150 155

CCA ACA TCG CTG TAC CAT CAG GCG ATG AAA GGT GTC AGA ACG GCG 608
 Pro Thr Ser Leu Tyr His Gln Ala Met Lys Gly Val Arg Thr Ala
 160 165 170

TAT TGG ATT GGG TTT GAC ACC CCG TTT ATG TTT GAC GCG CTA 653
 Tyr Trp Ile Gly Phe Asp Thr Thr Pro Phe Met Phe Asp Ala Leu
 175 180 185

GCA GGC GCG TAT CCA ACC TAC GCC ACA AAC TGG GCC GAC GAG CAG 698
 Ala Gly Ala Tyr Pro Thr Tyr Ala Thr Asn Trp Ala Asp Glu Gln
 190 195 200

GTG TTA CAG GCC AGG AAC ATA GGA CTG TGT GCA GCA TCC TTG ACT 743
 Val Leu Gln Ala Arg Asn Ile Gly Leu Cys Ala Ala Ser Leu Thr
 205 210 215

GAG GGA AGA CTC GGC AAA CTG TCC ATT CTC CGC AAG AAG CAA TTG 788
 Glu Gly Arg Leu Gly Lys Leu Ser Ile Leu Arg Lys Lys Gln Leu
 220 225 230

AAA CCT TGC GAC ACA GTC ATG TTC TCG GTA GGA TCT ACA TTG TAC 833
 Lys Pro Cys Asp Thr Val Met Phe Ser Val Gly Ser Thr Leu Tyr
 235 240 245

ACT GAG AGC AGA AAG CTA CTG AGG AGC TGG CAC TTA CCC TCC GTA 878
 Thr Glu Ser Arg Lys Leu Leu Arg Ser Trp His Leu Pro Ser Val
 250 255 260

TTC CAC CTG AAA GGT AAA CAA TCC TTT ACC TGT AGG TGC GAT ACC 923
 Phe His Leu Lys Gly Lys Gln Ser Phe Thr Cys Arg Cys Asp Thr
 265 270 275

ATC GTA TCA TGT GAA GGG TAC GTA GTT AAG AAA ATC ACT ATG TGC 968
 Ile Val Ser Cys Glu Gly Tyr Val Val Lys Lys Ile Thr Met Cys
 280 285 290

CCC GGC CTG TAC GGT AAA ACG GTA GGG TAC GCC GTG ACG TAT CAC 1013
 Pro Gly Leu Tyr Gly Lys Thr Val Gly Tyr Ala Val Thr Tyr His
 295 300 305

GCG GAG GGA TTC CTA GTG TGC AAG ACC ACA GAC ACT GTC AAA GGA 1058
 Ala Glu Gly Phe Leu Val Cys Lys Thr Thr Asp Thr Val Lys Gly
 310 315 320

GAA AGA GTC TCA TTC CCT GTA TGC ACC TAC GTC CCC TCA ACC ATC 1103
 Glu Arg Val Ser Phe Pro Val Cys Thr Tyr Val Pro Ser Thr Ile
 325 330 335

TGT GAT CAA ATG ACT GGC ATA CTA GCG ACC GAC GTC ACA CCG GAG 1148
 Cys Asp Gln Met Thr Gly Ile Leu Ala Thr Asp Val Thr Pro Glu
 340 345 350

GAC GCA CAG AAG TTG TTA GTG GGA TTG AAT CAG AGG ATA GTT GTG 1193
 Asp Ala Gln Lys Leu Leu Val Gly Leu Asn Gln Arg Ile Val Val
 355 360 365

AAC GGA AGA ACA CAG CGA AAC ACT AAC ACG ATG AAG AAC TAT CTG 1238
 Asn Gly Arg Thr Gln Arg Asn Thr Asn Thr Met Lys Asn Tyr Leu
 370 375 380

CTT CCG ATT GTG GCC GTC GCA TTT AGC AAG TGG GCG AGG GAA TAC 1283
 Leu Pro Ile Val Ala Val Ala Phe Ser Lys Trp Ala Arg Glu Tyr
 385 390 395

AAG GCA GAC CTT GAT GAT GAA AAA CCT CTG GGT GTC CGA GAG AGG 1328
 Lys Ala Asp Leu Asp Asp Glu Lys Pro Leu Gly Val Arg Glu Arg
 400 405 410

TCA CTT ACT TGC TGC TTG TGG GCA TTT AAA ACG AGG AAG ATG 1373
 Ser Leu Thr Cys Cys Leu Trp Ala Phe Lys Thr Arg Lys Met
 415 420 425

CAC ACC ATG TAC AAG AAA CCA GAC ACC CAG ACA ATA GTG AAG GTG 1418
 His Thr Met Tyr Lys Lys Pro Asp Thr Gln Thr Ile Val Lys Val
 430 435 440

CCT TCA GAG TTT AAC TCG TTC GTC ATC CCG AGC CTA TGG TCT ACA 1463
 Pro Ser Glu Phe Asn Ser Phe Val Ile Pro Ser Leu Trp Ser Thr
 445 450 455

GGC CTC GCA ATC CCA GTC AGA TCA CGC ATT AAG ATG CTT TTG GCC 1508
 Gly Leu Ala Ile Pro Val Arg Ser Arg Ile Lys Met Leu Leu Ala
 460 465 470

AAG AAG ACC AAG CGA GAG TTA ATA CCT GTT CTC GAC GCG TCG TCA 1553
 Lys Lys Thr Lys Arg Glu Leu Ile Pro Val Leu Asp Ala Ser Ser
 475 480 485

GCC AGG GAT GCT GAA CAA GAG GAG AAG GAG AGG TTG GAG GCC GAG 1598
 Ala Arg Asp Ala Glu Gln Glu Glu Lys Glu Arg Leu Glu Ala Glu
 490 495 500

CTG ACT AGA GAA GCC TTA CCA CCC CTC GTC CCC ATC GCG CCG GCG 1643
 Leu Thr Arg Glu Ala Leu Pro Pro Leu Val Pro Ile Ala Pro Ala
 505 510 515

GAG ACG GGA GTC GTC GAC GTC GAC GTT GAA GAA CTA GAG TAT CAC 1688
 Glu Thr Gly Val Val Asp Val Asp Val Glu Glu Leu Glu Tyr His
 520 525 530

GCA GGT GCA GGG GTC GTG GAA ACA CCT CGC AGC GCG TTG AAA GTC 1733
 Ala Gly Ala Gly Val Val Glu Thr Pro Arg Ser Ala Leu Lys Val
 535 540 545

ACC GCA CAG CCG AAC GAC GTA CTA CTA GGA AAT TAC GTA GTT CTG 1778
 Thr Ala Gln Pro Asn Asp Val Leu Leu Gly Asn Tyr Val Val Leu
 550 555 560

TCC CCG CAG ACC GTG CTC AAG AGC TCC AAG TTG GCC CCC GTG CAC 1823
 Ser Pro Gln Thr Val Leu Lys Ser Ser Lys Leu Ala Pro Val His
 565 570 575

CCT CTA GCA GAG CAG GTG AAA ATA ATA ACA CAT AAC GGG AGG GCC 1868
 Pro Leu Ala Glu Gln Val Lys Ile Ile Thr His Asn Gly Arg Ala
 580 585 590

GGC GGT TAC CAG GTC GAC GGA TAT GAC GGC AGG GTC CTA CTA CCA 1913
 Gly Gly Tyr Gln Val Asp Gly Tyr Asp Gly Arg Val Leu Leu Pro
 595 600 605

TGT GGA TCG GCC ATT CCG GTC CCT GAG TTT CAA GCT TTG AGC GAG 1958
 Cys Gly Ser Ala Ile Pro Val Pro Glu Phe Gln Ala Leu Ser Glu
 610 615 620

AGC GCC ACT ATG GTG TAC AAC GAA AGG GAG TTC GTC AAC AGG AAA 2003
 Ser Ala Thr Met Val Tyr Asn Glu Arg Glu Phe Val Asn Arg Lys
 625 630 635

CTA TAC CAT ATT GCC GTT CAC GGA CCG TCG CTG AAC ACC GAC GAG 2048
 Leu Tyr His Ile Ala Val His Gly Pro Ser Leu Asn Thr Asp Glu
 640 645 650

GAG AAC TAC GAG AAA GTC AGA GCT GAA AGA ACT GAC GCC GAG TAC 2093
 Glu Asn Tyr Glu Lys Val Arg Ala Glu Arg Thr Asp Ala Glu Tyr
 655 660 665

GTG TTC GAC GTA GAT AAA AAA TGC TGC GTC AAG AGA GAG GAA GCG 2138
 Val Phe Asp Val Asp Lys Lys Cys Cys Val Lys Arg Glu Glu Ala
 670 675 680

TCG GGT TTG GTG TTG GTG GGA GAG CTA ACC AAC CCC CCG TTC CAT 2183
 Ser Gly Leu Val Leu Val Gly Glu Leu Thr Asn Pro Pro Phe His
 685 690 695

GAA TTC GCC TAC GAA GGG CTG AAG ATC AGG CCG TCG GCA CCA TAT 2228
 Glu Phe Ala Tyr Glu Gly Leu Lys Ile Arg Pro Ser Ala Pro Tyr
 700 705 710

AAG ACT ACA GTA GTA GGA GTC TTT GGG GTT CCG GGA TCA GGC AAG 2273
 Lys Thr Thr Val Val Gly Val Phe Gly Val Pro Gly Ser Gly Lys
 715 720 725

TCT GCT ATT ATT AAG AGC CTC GTG ACC AAA CAC GAT CTG GTC ACC 2318
 Ser Ala Ile Ile Lys Ser Leu Val Thr Lys His Asp Leu Val Thr
 730 735 740

AGC GGC AAG AAG GAG AAC TGC CAG GAA ATA GTT AAC GAC GTG AAG 2363
 Ser Gly Lys Lys Glu Asn Cys Gln Glu Ile Val Asn Asp Val Lys
 745 750 755

AAG CAC CGC GGG AAG GGG ACA AGT AGG GAA AAC AGT GAC TCC ATC 2408
 Lys His Arg Gly Lys Gly Thr Ser Arg Glu Asn Ser Asp Ser Ile
 760 765 770

CTG CTA AAC GGG TGT CGT CGT GCC GTG GAC ATC CTA TAT GTG GAC 2453
 Leu Leu Asn Gly Cys Arg Arg Ala Val Asp Ile Leu Tyr Val Asp
 775 780 785

GAG CCT TTC GCT TGC CAT TCC GGT ACT CTG CTG GCC CTA ATT GCT 2498
 Glu Ala Phe Ala Cys His Ser Gly Thr Leu Leu Ala Leu Ile Ala
 790 795 800

CTT GTT AAA CCT CGG AGC AAA GTG GTG TTA TGC GGA GAC CCC AAG 2543
 Leu Val Lys Pro Arg Ser Lys Val Val Leu Cys Gly Asp Pro Lys
 805 810 815

CAA TGC GGA TTC TTC AAT ATG ATG CAG CTT AAG GTG AAC TTC AAC 2588
 Gln Cys Gly Phe Phe Asn Met Met Gln Leu Lys Val Asn Phe Asn
 820 825 830

CAC AAC ATC TGC ACT GAA GTA TGT CAT AAA AGT ATA TCC AGA CGT 2633
 His Asn Ile Cys Thr Glu Val Cys His Lys Ser Ile Ser Arg Arg
 835 840 845

TGC ACC CGT CCA GTC ACG GCC ATC GTG TCT ACG TTG CAC TAC GGA 2678
 Cys Thr Arg Pro Val Thr Ala Ile Val Ser Thr Leu His Tyr Gly
 850 855 860

GGC AAG ATG CGC ACG ACC AAC CCG TGC AAC AAA CCC ATA ATC ATA 2723
 Gly Lys Met Arg Thr Thr Asn Pro Cys Asn Lys Pro Ile Ile Ile
 865 870 875

GAC ACC ACA GGA CAG ACC AAG CCC AAG CCA GGA GAC ATC GTG TTA 2768
 Asp Thr Thr Gly Gln Thr Lys Pro Lys Pro Gly Asp Ile Val Leu
 880 885 890

ACA TGC TTC CGA GGC TGG GCA AAG CAG CTG CAG TTG GAC TAC CGT 2813
 Thr Cys Phe Arg Gly Trp Ala Lys Gln Leu Gln Leu Asp Tyr Arg
 895 900 905

GGA CAC GAA GTC ATG ACA GCA GCA TCT CAG GGC CTC ACC CGC 2858
 Gly His Glu Val Met Thr Ala Ala Ser Gln Gly Leu Thr Arg
 910 915 920

AAA GGG GTA TAC GCC GTA AGG CAG AAG GTG AAT GAA AAT CCC TTG 2903
 Lys Gly Val Tyr Ala Val Arg Gln Lys Val Asn Glu Asn Pro Leu
 925 930 935

TAT GCC CCT GCG TCG GAG CAC GTG AAT GTA CTG CTG ACG CGC ACT 2948
 Tyr Ala Pro Ala Ser Glu His Val Asn Val Leu Leu Thr Arg Thr
 940 945 950

GAG GAT AGG CTG GTG TGG AAA ACG CTG GCC GGC GAT CCC TGG ATT 2993
 Glu Asp Arg Leu Val Trp Lys Thr Leu Ala Gly Asp Pro Trp Ile
 955 960 965

AAG GTC CTA TCA AAC ATT CCA CAG GGT AAC TTT ACG GCC ACA TTG 3038
 Lys Val Leu Ser Asn Ile Pro Gln Gly Asn Phe Thr Ala Thr Leu
 970 975 980

GAA GAA TGG CAA GAA GAA CAC GAC AAA ATA ATG AAG GTG ATT GAA 3083
 Glu Glu Trp Gln Glu His Asp Lys Ile Met Lys Val Ile Glu
 985 990 995

GGA CCG GCT GCG CCT GTG GAC GCG TTC CAG AAC AAA GCG AAC GTG 3128
 Gly Pro Ala Ala Pro Val Asp Ala Phe Gln Asn Lys Ala Asn Val
 1000 1005 1010

TGT TGG GCG AAA AGC CTG GTG CCT GTC CTG GAC ACT GCC GGA ATC 3173
 Cys Trp Ala Lys Ser Leu Val Pro Val Leu Asp Thr Ala Gly Ile
 1015 1020 1025

Figure 5 (6)

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AGA TTG ACA GCA GAG GAG TGG AGC ACC ATA ATT ACA GCA TTT AAG 3218
 Arg Leu Thr Ala Glu Glu Trp Ser Thr Ile Ile Thr Ala Phe Lys
 1030 1035 1040

GAG GAC AGA GCT TAC TCT CCA GTG GTG GCC TTG AAT GAA ATT TGC 3263
 Glu Asp Arg Ala Tyr Ser Pro Val Val Ala Leu Asn Glu Ile Cys
 1045 1050 1055

ACC AAG TAC TAT GGA GTT GAC CTG GAC AGT GGC CTG TTT TCT GCC 3308
 Thr Lys Tyr Tyr Gly Val Asp Leu Asp Ser Gly Leu Phe Ser Ala
 1060 1065 1070

CCG AAG GTG TCC CTG TAT TAC GAG AAC AAC CAC TGG GAT AAC AGA 3353
 Pro Lys Val Ser Leu Tyr Tyr Glu Asn Asn His Trp Asp Asn Arg
 1075 1080 1085

CCT GGT GGA AGG ATG TAT GGA TTC AAT GCC GCA ACA GCT GCC AGG 3398
 Pro Gly Gly Arg Met Tyr Gly Phe Asn Ala Ala Thr Ala Ala Arg
 1090 1095 1100

CTG GAA GCT AGA CAT ACC TTC CTG AAG GGG CAG TGG CAT ACG GGC 3443
 Leu Glu Ala Arg His Thr Phe Leu Lys Gly Gln Trp His Thr Gly
 1105 1110 1115

AAG CAG GCA GTT ATC GCA GAA AGA AAA ATC CAA CCG CTT TCT GTG 3488
 Lys Gln Ala Val Ile Ala Glu Arg Lys Ile Gln Pro Leu Ser Val
 1120 1125 1130

CTG GAC AAT GTA ATT CCT ATC AAC CGC AGG CTG CCG CAC GCC CTG 3533
 Leu Asp Asn Val Ile Pro Ile Asn Arg Arg Leu Pro His Ala Leu
 1135 1140 1145

GTG GCT GAG TAC AAG ACG GTT AAA GGC AGT AGG GTT GAG TGG CTG 3578
 Val Ala Glu Tyr Lys Thr Val Lys Gly Ser Arg Val Glu Trp Leu
 1150 1155 1160

GTC AAT AAA GTA AGA GGG TAC CAC GTC CTG CTG GTG AGT GAG TAC 3623
 Val Asn Lys Val Arg Gly Tyr His Val Leu Leu Val Ser Glu Tyr
 1165 1170 1175

AAC CTG GCT TTG CCT CGA CGC AGG GTC ACT TGG TTG TCA CCG CTG 3668
 Asn Leu Ala Leu Pro Arg Arg Val Thr Trp Leu Ser Pro Leu
 1180 1185 1190

AAT GTC ACA GGC GCC GAT AGG TGC TAC GAC CTA AGT TTA GGA CTG 3713
 Asn Val Thr Gly Ala Asp Arg Cys Tyr Asp Leu Ser Leu Gly Leu
 1195 1200 1205

CCG GCT GAC GCC GGC AGG TTC GAC TTG GTC TTT GTG AAC ATT CAC 3758
 Pro Ala Asp Ala Gly Arg Phe Asp Leu Val Phe Val Asn Ile His
 1210 1215 1220

ACG GAA TTC AGA ATC CAC CAC TAC CAG CAG TGT GTC GAC CAC GCC 3803
 Thr Glu Phe Arg Ile His His Tyr Gln Gln Cys Val Asp His Ala
 1225 1230 1235

ATG AAG CTG CAG ATG CTT GGG GGA GAT GCG CTA CGA CTG CTA AAA 3848
 Met Lys Leu Gln Met Leu Gly Gly Asp Ala Leu Arg Leu Leu Lys
 1240 1245 1250

CCC GGC GGC ATC TTG ATG AGA GCT TAC GGA TAC GCC GAT AAA ATC 3893
 Pro Gly Gly Ile Leu Met Arg Ala Tyr Gly Tyr Ala Asp Lys Ile
 1255 1260 1265

AGC GAA GCC GTT GTT TCC TCC TTA AGC AGA AAG TTC TCG TCT GCA 3938
 Ser Glu Ala Val Val Ser Ser Leu Ser Arg Lys Phe Ser Ser Ala
 1270 1275 1280

AGA GTG TTG CCG CCG GAT TGT GTC ACC AGC AAT ACA GAA GTG TTC 3983
 Arg Val Leu Arg Pro Asp Cys Val Thr Ser Asn Thr Glu Val Phe
 1285 1290 1295

TTG CTG TTC TCC AAC TTT GAC AAC GGA AAG AGA CCC TCT ACG CTA 4028
 Leu Leu Phe Ser Asn Phe Asp Asn Gly Lys Arg Pro Ser Thr Leu
 1300 1305 1310

CAC CAG ATG AAT ACC AAG CTG AGT GCC GTG TAT GCC GGA GAA GCC 4073
 His Gln Met Asn Thr Lys Leu Ser Ala Val Tyr Ala Gly Glu Ala
 1315 1320 1325

ATG CAC ACG GCC GGG TGT GCA CCA TCC TAC AGA GTT AAG AGA GCA 4118
 Met His Thr Ala Gly Cys Ala Pro Ser Tyr Arg Val Lys Arg Ala
 1330 1335 1340

GAC ATA GCC ACG TGC ACA GAA GCG GCT GTG GTT AAC GCA GCT AAC 4163
 Asp Ile Ala Thr Cys Thr Glu Ala Ala Val Val Asn Ala Ala Asn
 1345 1350 1355

GCC CGT GGA ACT GTA GGG GAT GGC GTA TGC AGG GCC GTG GCG AAG 4208
 Ala Arg Gly Thr Val Gly Asp Gly Val Cys Arg Ala Val Ala Lys
 1360 1365 1370

AAA TGG CCG TCA GCC TTT AAG GGA GCA GCA ACA CCA GTG GGC ACA 4253
 Lys Trp Pro Ser Ala Phe Lys Gly Ala Ala Thr Pro Val Gly Thr
 1375 1380 1385

ATT AAA ACA GTC ATG TGC GGC TCG TAC CCC GTC ATC CAC GCT GTA 4298
 Ile Lys Thr Val Met Cys Gly Ser Tyr Pro Val Ile His Ala Val
 1390 1395 1400

GCG CCT AAT TTC TCT GCC ACG ACT GAA GCG GAA GGG GAC CGC GAA 4343
 Ala Pro Asn Phe Ser Ala Thr Thr Glu Ala Glu Gly Asp Arg Glu
 1405 1410 1415

TTG GCC GCT GTC TAC CGG GCA GTG GCC GAA GTA AAC AGA CTG 4388
 Leu Ala Ala Val Tyr Arg Ala Val Ala Glu Val Asn Arg Leu
 1420 1425 1430

TCA CTG AGC AGC GTA GCC ATC CCG CTG CTG TCC ACA GGA GTG TTC 4433
 Ser Leu Ser Ser Val Ala Ile Pro Leu Leu Ser Thr Gly Val Phe
 1435 1440 1445

AGC GGC GGA AGA GAT AGG CTG CAG CAA TCC CTC AAC CAT CTA TTC 4478
 Ser Gly Gly Arg Asp Arg Leu Gln Gln Ser Leu Asn His Leu Phe
 1450 1455 1460

ACA GCA ATG GAC GCC ACG GAC GCT GAC GTG ACC ATC TAC TGC AGA 4523
 Thr Ala Met Asp Ala Thr Asp Ala Asp Val Thr Ile Tyr Cys Arg
 1465 1470 1475

GAC AAA AGT TGG GAG AAG AAA ATC CAG GAA GCC ATT GAC ATG AGG 4568
 Asp Lys Ser Trp Glu Lys Lys Ile Gln Glu Ala Ile Asp Met Arg
 1480 1485 1490

ACG GCT GTG GAG TTG CTC AAT GAT GAC GTG GAG CTG ACC ACA GAC 4613
 Thr Ala Val Glu Leu Leu Asn Asp Asp Val Glu Leu Thr Thr Asp
 1495 1500 1505

TTG GTG AGA GTG CAC CCG GAC AGC AGC CTG GTG GGT CGT AAG GGC 4658
 Leu Val Arg Val His Pro Asp Ser Ser Leu Val Gly Arg Lys Gly
 1510 1515 1520

TAC AGT ACC ACT GAC GGG TCG CTG TAC TCG TAC TTT GAA GGT ACG 4703
 Tyr Ser Thr Thr Asp Gly Ser Leu Tyr Ser Tyr Phe Glu Gly Thr
 1525 1530 1535

AAA TTC AAC CAG GCT ATT GAT ATG GCA GAG ATA CTG ACG TTG 4748
 Lys Phe Asn Gln Ala Ala Ile Asp Met Ala Glu Ile Leu Thr Leu
 1540 1545 1550

TGG CCC AGA CTG CAA GAG GCA AAC GAA CAG ATA TGC CTA TAC GCG 4793
 Trp Pro Arg Leu Gln Glu Ala Asn Glu Gln Ile Cys Leu Tyr Ala
 1555 1560 1565

CTG GGC GAA ACA ATG GAC AAC ATC AGA TCC AAA TGT CCG GTG AAC 4838
 Leu Gly Glu Thr Met Asp Asn Ile Arg Ser Lys Cys Pro Val Asn
 1570 1575 1580

GAT TCC GAT TCA TCA ACA CCT CCC AGG ACA GTG CCC TGC CTG TGC 4883
 Asp Ser Asp Ser Ser Thr Pro Pro Arg Thr Val Pro Cys Leu Cys
 1585 1590 1595

CGC TAC GCA ATG ACA GCA GAA CGG ATC GCC CGC CTT AGG TCA CAC 4928
 Arg Tyr Ala Met Thr Ala Glu Arg Ile Ala Arg Leu Arg Ser His
 1600 1605 1610

CAA GTT AAA AGC ATG GTG GTT TGC TCA TCT TTT CCC CTC CCG AAA 4973
 Gln Val Lys Ser Met Val Val Cys Ser Ser Phe Pro Leu Pro Lys
 1615 1620 1625

TAC CAT GTA GAT GGG GTG CAG AAG GTA AAG TGC GAG AAG GTT CTC 5018
 Tyr His Val Asp Gly Val Gln Lys Val Lys Cys Glu Lys Val Leu
 1630 1635 1640

CTG TTC GAC CCG ACG GTA CCT TCA GTG GTT AGT CCG CGG AAG TAT 5063
 Leu Phe Asp Pro Thr Val Pro Ser Val Val Ser Pro Arg Lys Tyr
 1645 1650 1655

GCC GCA TCT ACG ACG GAC CAC TCA GAT CGG TCG TTA CGA GGG TTT 5108
 Ala Ala Ser Thr Thr Asp His Ser Asp Arg Ser Leu Arg Gly Phe
 1660 1665 1670

GAC TTG GAC TGG ACC ACC GAC TCG TCT TCC ACT GCC AGC GAT ACC 5153
 Asp Leu Asp Trp Thr Thr Asp Ser Ser Ser Thr Ala Ser Asp Thr
 1675 1680 1685

ATG TCG CTA CCC AGT TTG CAG TCG TGT GAC ATC GAC TCG ATC TAC 5198
 Met Ser Leu Pro Ser Leu Gln Ser Cys Asp Ile Asp Ser Ile Tyr
 1690 1695 1700

GAG CCA ATG GCT CCC ATA GTA GTG ACG GCT GAC GTA CAC CCT GAA 5243
 Glu Pro Met Ala Pro Ile Val Val Thr Ala Asp Val His Pro Glu
 1705 1710 1715

CCC GCA GGC ATC GCG GAC CTG GCG GCA GAT GTG CAC CCT GAA CCC 5288
 Pro Ala Gly Ile Ala Asp Leu Ala Ala Asp Val His Pro Glu Pro
 1720 1725 1730

GCA GAC CAT GTG GAC CTC GAG AAC CCG ATT CCT CCA CCG CGC CCG 5333
 Ala Asp His Val Asp Leu Glu Asn Pro Ile Pro Pro Pro Arg Pro
 1735 1740 1745

AAG AGA GCT GCA TAC CTT GCC TCC CGC GCG GCG GAG CGA CCG GTG 5378
 Lys Arg Ala Ala Tyr Leu Ala Ser Arg Ala Ala Glu Arg Pro Val
 1750 1755 1760

CCG GCG CCG AGA AAG CCG ACG CCT GCC CCA AGG ACT GCG TTT AGG 5423
 Pro Ala Pro Arg Lys Pro Thr Pro Ala Pro Arg Thr Ala Phe Arg
 1765 1770 1775

AAC AAG CTG CCT TTG ACG TTC GGC GAC TTT GAC GAG CAC GAG GTC 5468
 Asn Lys Leu Pro Leu Thr Phe Gly Asp Phe Asp Glu His Glu Val
 1780 1785 1790

GAT GCG TTG GCC TCC GGG ATT ACT TTC GGA GAC TTC GAC GAC GTC 5513
 Asp Ala Leu Ala Ser Gly Ile Thr Phe Gly Asp Phe Asp Asp Asp Val
 1795 1800 1805

CTG CGA CTA GGC CGC GCG GGT GCA TAT ATT TTC TCC TCG GAC ACT 5558
 Leu Arg Leu Gly Arg Ala Gly Ala Tyr Ile Phe Ser Ser Asp Thr
 1810 1815 1820

GGC AGC GGA CAT TTA CAA CAA AAA TCC GTT AGG CAG CAC AAT CTC 5603
 Gly Ser Gly His Leu Gln Gln Lys Ser Val Arg Gln His Asn Leu
 1825 1830 1835

CAG TGC GCA CAA CTG GAT GCG GTC CAG GAG GAG AAA ATG TAC CCG 5648
 Gln Cys Ala Gln Leu Asp Ala Val Gln Glu Glu Lys Met Tyr Pro
 1840 1845 1850

CCA AAA TTG GAT ACT GAG AGG GAG AAG CTG TTG CTG CTG AAA ATG 5693
 Pro Lys Leu Asp Thr Glu Arg Glu Lys Leu Leu Leu Lys Met
 1855 1860 1865

Figure 5 (10)

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CAG ATG CAC CCA TCG GAG GCT AAT AAG AGT CGA TAC CAG TCT CGC 5738
 Gln Met His Pro Ser Glu Ala Asn Lys Ser Arg Tyr Gln Ser Arg
 1870 1875 1880

AAA GTG GAG AAC ATG AAA GCC ACG GTG GTG GAC AGG CTC ACA TCG 5783
 Lys Val Glu Asn Met Lys Ala Thr Val Val Asp Arg Leu Thr Ser
 1885 1890 1895

GGG GCC AGA TTG TAC ACG GGA GCG GAC GTA GGC CGC ATA CCA ACA 5828
 Gly Ala Arg Leu Tyr Thr Gly Ala Asp Val Gly Arg Ile Pro Thr
 1900 1905 1910

TAC GCG GTT CGG TAC CCC CGC CCC GTG TAC TCC CCT ACC GTG ATC 5873
 Tyr Ala Val Arg Tyr Pro Arg Pro Val Tyr Ser Pro Thr Val Ile
 1915 1920 1925

GAA AGA TTC TCA AGC CCC GAT GTA GCA ATC GCA GCG TGC AAC GAA 5918
 Glu Arg Phe Ser Ser Pro Asp Val Ala Ile Ala Ala Cys Asn Glu
 1930 1935 1940

TAC CTA TCC AGA AAT TAC CCA ACA GTG GCG TCG TAC CAG ATA ACA 5963
 Tyr Leu Ser Arg Asn Tyr Pro Thr Val Ala Ser Tyr Gln Ile Thr
 1945 1950 1955

GAT GAA TAC GAC GCA TAC TTG GAC ATG GTT GAC GGG TCG GAT AGT 6008
 Asp Glu Tyr Asp Ala Tyr Leu Asp Met Val Asp Gly Ser Asp Ser
 1960 1965 1970

TGC TTG GAC AGA GCG ACA TTC TGC CCG GCG AAG CTC CGG TGC TAC 6053
 Cys Leu Asp Arg Ala Thr Phe Cys Pro Ala Lys Leu Arg Cys Tyr
 1975 1980 1985

CCG AAA CAT CAT GCG TAC CAC CAG CCG ACT GTA CGC AGT GCC GTC 6098
 Pro Lys His His Ala Tyr His Gln Pro Thr Val Arg Ser Ala Val
 1990 1995 2000

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 Pro Ser Pro Phe Gln Asn Thr Leu Gln Asn Val Leu Ala Ala Ala
 2005 2010 2015

ACC AAG AGA AAC TGC AAC GTC ACG CAA ATG CGA GAA CTA CCC ACC 6188
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 2020 2025 2030

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 2035 2040 2045

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 Cys Ser Gly Glu Tyr Trp Glu Glu Tyr Ala Lys Gln Pro Ile Arg
 2050 2055 2060

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 2065 2070 2075

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 Pro Lys Ala Ala Ala Leu Phe Ala Lys Thr His Asn Leu Val Pro
 2080 2085 2090

CTG CAG GAG GTT CCC ATG GAC AGA TTC ACG GTC GAC ATG AAA CGA 6413
 Leu Gln Glu Val Pro Met Asp Arg Phe Thr Val Asp Met Lys Arg
 2095 2100 2105

GAT GTC AAA GTC ACT CCA GGG ACG AAA CAC ACA GAG GAA AGA CCC 6458
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 2110 2115 2120

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 Lys Val Gln Val Ile Gln Ala Ala Glu Pro Leu Ala Thr Ala Tyr
 2125 2130 2135

CTG TGC GGC ATC CAC AGG GAA TTA GTA AGG AGA CTA AAT GCT GTG 6548
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 2140 2145 2150

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 Leu Arg Pro Asn Val His Thr Leu Phe Asp Met Ser Ala Glu Asp
 2155 2160 2165

TTT GAC GCG ATC ATC GCC TCT CAC TTC CAC CCA GGA GAC CCG GTT 6638
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 2170 2175 2180

CTA GAG ACG GAC ATT GCA TCA TTC GAC AAA AGC CAG GAC GAC TCC 6683
 Leu Glu Thr Asp Ile Ala Ser Phe Asp Lys Ser Gln Asp Asp Ser
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 2200 2205 2210

CAG TAC CTG CTG GAC TTG ATC GAG GCA GCC TTT GGG GAA ATA TCC 6773
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 Met Lys Ser Gly Met Phe Leu Thr Leu Phe Ile Asn Thr Val Leu
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 Asn Ile Thr Ile Ala Ser Arg Val Leu Glu Gln Arg Leu Thr Asp
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TCC GCC TGT GCG GCC TTC ATC GGC GAC AAC ATC GTT CAC GGA 6953
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 Val Ile Ser Asp Lys Leu Met Ala Glu Arg Cys Ala Ser Trp Val
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 2350 2355 2360

CGA GCA CTG AGT GAC GAG GTT AGC AAG TGG TTC CCG ACA GGC TTG 7223
 Arg Ala Leu Ser Asp Glu Val Ser Lys Trp Phe Arg Thr Gly Leu
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 Gly Ala Glu Leu Glu Val Ala Leu Thr Ser Arg Tyr Glu Val Glu
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 Gly Cys Lys Ser Ile Leu Ile Ala Met Thr Thr Leu Ala Arg Asp
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 60 65 70

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 Cys Leu Val Gly Asp Lys Val Met Lys Pro Ala His Val Lys Gly
 135 140 145

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 Val Ile Asp Asn Ala Asp Leu Ala Lys Leu Ala Phe Lys Lys Ser
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 Ser Lys Tyr Asp Leu Glu Cys Ala Gln Ile Pro Val His Met Arg
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 Ser Asp Ala Ser Lys Tyr Thr His Glu Lys Pro Glu Gly His Tyr
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 Asn Trp His His Gly Ala Val Gln Tyr Ser Gly Gly Arg Phe Thr
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 Phe Asp Asn Lys Gly Arg Val Val Ala Ile Val Leu Gly Gly Ala
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 Asn Glu Gly Ser Arg Thr Ala Leu Ser Val Val Thr Trp Asn Lys
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 Asp Met Val Thr Arg Val Thr Pro Glu Gly Ser Glu Glu Trp Ser
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 Ala Pro Leu Ile Thr Ala Met Cys Val Leu Ala Asn Ala Thr Phe
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CCG TGC TTC CAG CCC CCG TGT GTA CCT TGC TGC TAT GAA AAC AAC 8314
 Pro Cys Phe Gln Pro Pro Cys Val Pro Cys Cys Tyr Glu Asn Asn
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CAC TCG TGT CAT AGC CCC GTA GCA ATT GAA GCG GTC AGG TCC GAA 8539
 His Ser Cys His Ser Pro Val Ala Ile Glu Ala Val Arg Ser Glu
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 Ala Thr Asp Gly Met Leu Lys Ile Gln Phe Ser Ala Gln Ile Gly
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 Gln Asp Thr Arg Asn Ala Val Arg Ala Cys Arg Ile Gln Tyr His
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 His Tyr Gly Lys Glu Ile Pro Cys Thr Thr Tyr Gln Gln Thr Thr
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GCG AAG ACC GTG GAG GAA ATC GAC ATG CAT ATG CCG CCA GAT ACG 8944
 Ala Lys Thr Val Glu Glu Ile Asp Met His Met Pro Pro Asp Thr
 495 500 505

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 Pro Asp Arg Thr Leu Leu Ser Gln Gln Ser Gly Asn Val Lys Ile
 510 515 520

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 Thr Val Gly Gly Lys Lys Val Lys Tyr Asn Cys Thr Cys Gly Thr
 525 530 535

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 Leu Ile Glu Gln Cys His Val Ser Val Thr Asp His Lys Lys Trp
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 Gln Phe Asn Ser Pro Phe Val Pro Arg Ala Asp Glu Pro Ala Arg
 570 575 580

AAA GGC AAA GTC CAT ATC CCA TTC CCG TTG GAC AAC ATC ACA TGC 9214
 Lys Gly Lys Val His Ile Pro Phe Pro Leu Asp Asn Ile Thr Cys
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 Glu Val Thr Leu His Leu His Pro Asp His Pro Thr Leu Phe Ser
 615 620 625

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 Tyr Arg Thr Leu Gly Glu Asp Pro Gln Tyr His Glu Glu Trp Val
 630 635 640

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 Thr Ala Ala Val Glu Arg Thr Ile Pro Val Pro Val Asp Gly Met
 645 650 655

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 Glu Tyr His Trp Gly Asn Asn Asp Pro Val Arg Leu Trp Ser Gln
 660 665 670

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 675 680 685

CAG TAC TAC TAT GGG CTT TAC CCG GCC GCT ACA GTA TCC GCG GTC 9529
 Gln Tyr Tyr Tyr Gly Leu Tyr Pro Ala Ala Thr Val Ser Ala Val
 690 695 700

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 705 710 715

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 735 740 745

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 Cys Ala Pro Arg Ala His Ala Ala Ser Val Ala Glu Thr Met Ala
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 795 800 805

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 Tyr Ser Pro Leu Thr Leu Gln Met Gln Val Val Glu Thr Ser Leu
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 Glu Pro Thr Leu Asn Leu Glu Tyr Ile Thr Cys Glu Tyr Lys Thr
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Figure 5 (17)

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AAC CCT GTC AGG GCC ATG AAC TGC GCC GTG GGA AAC ATC CCT GTC 10699
 Asn Pro Val Arg Ala Met Asn Cys Ala Val Gly Asn Ile Pro Val
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TCC ATG AAT TTG CCT GAC GCC TTT ACC CGC ATT GTC GAG GCG 10744
 Ser Met Asn Leu Pro Asp Ser Ala Phe Thr Arg Ile Val Glu Ala
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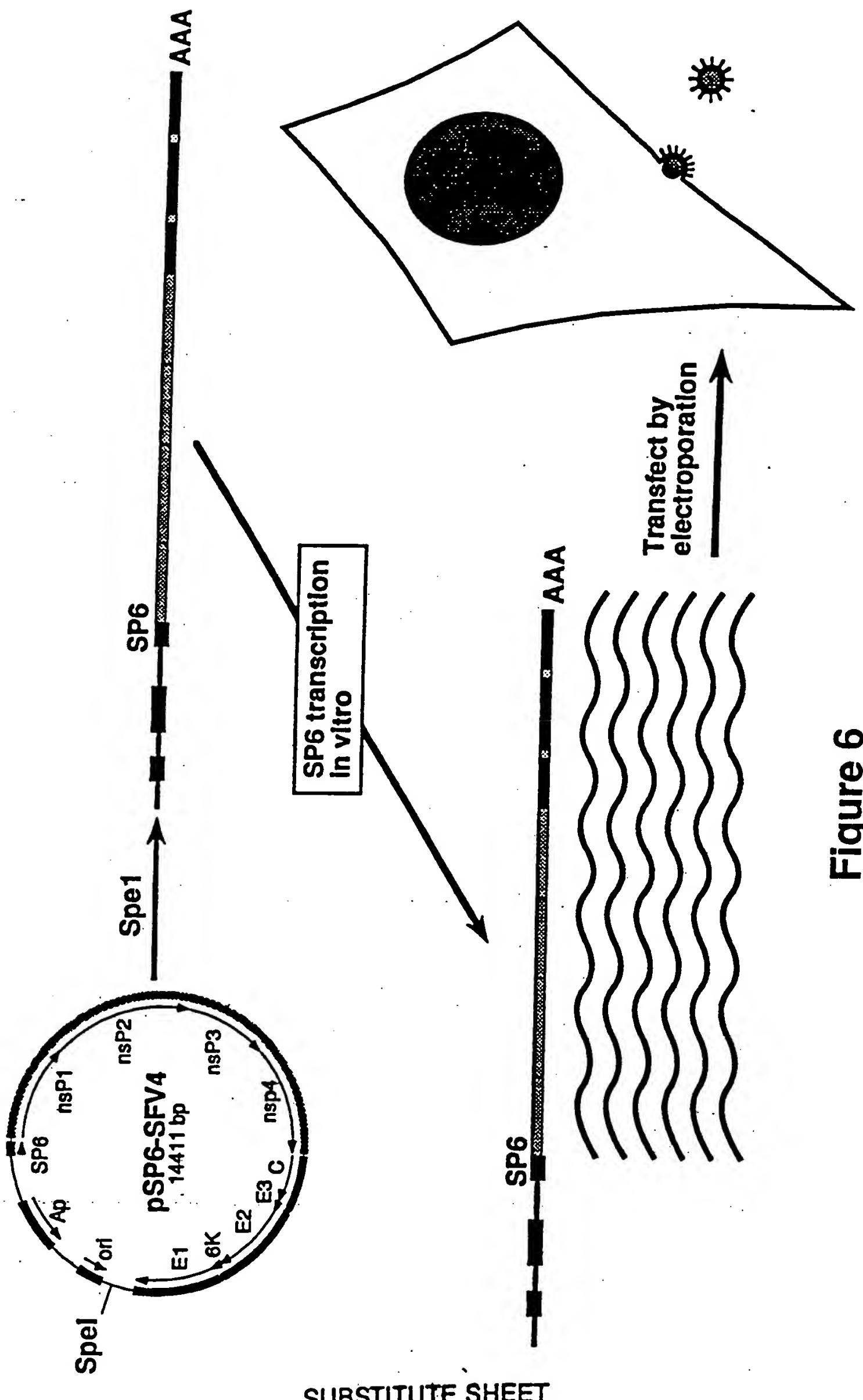


Figure 6

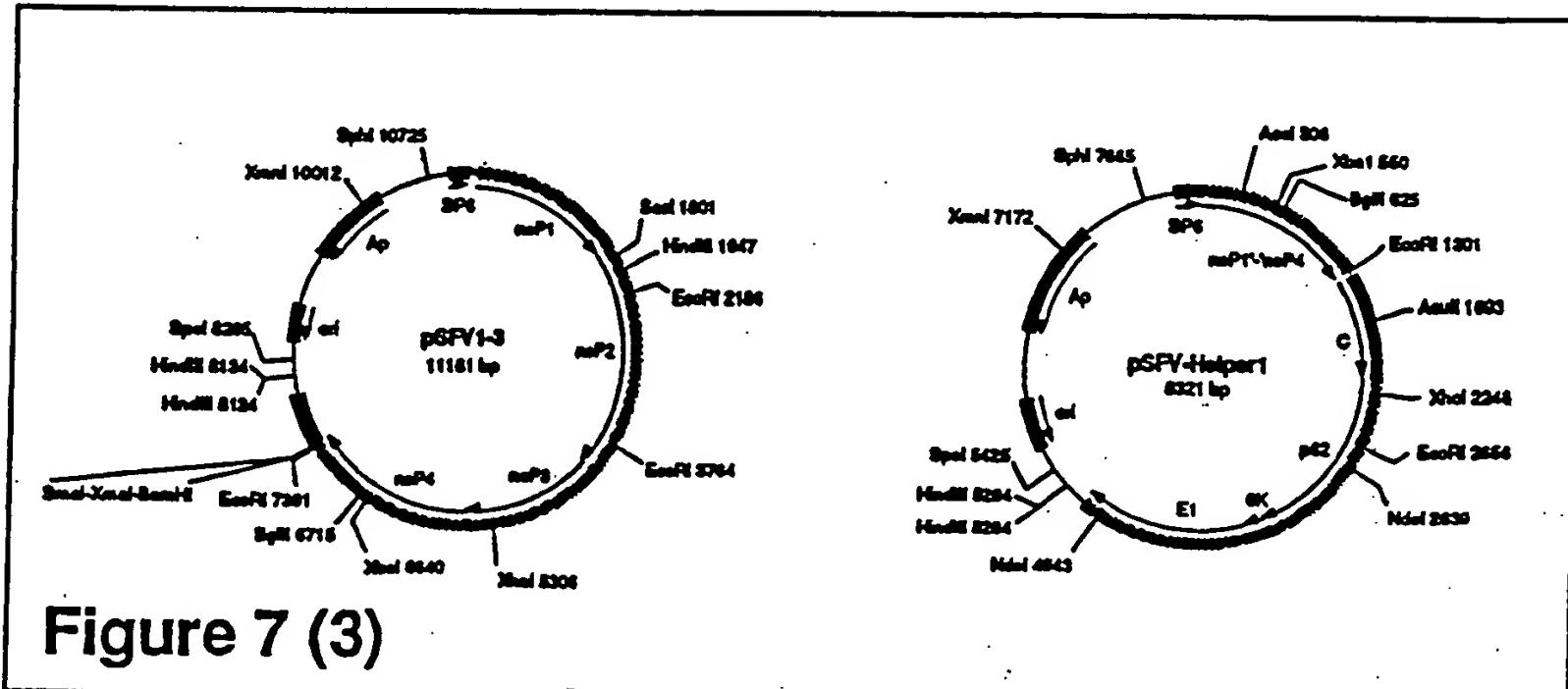
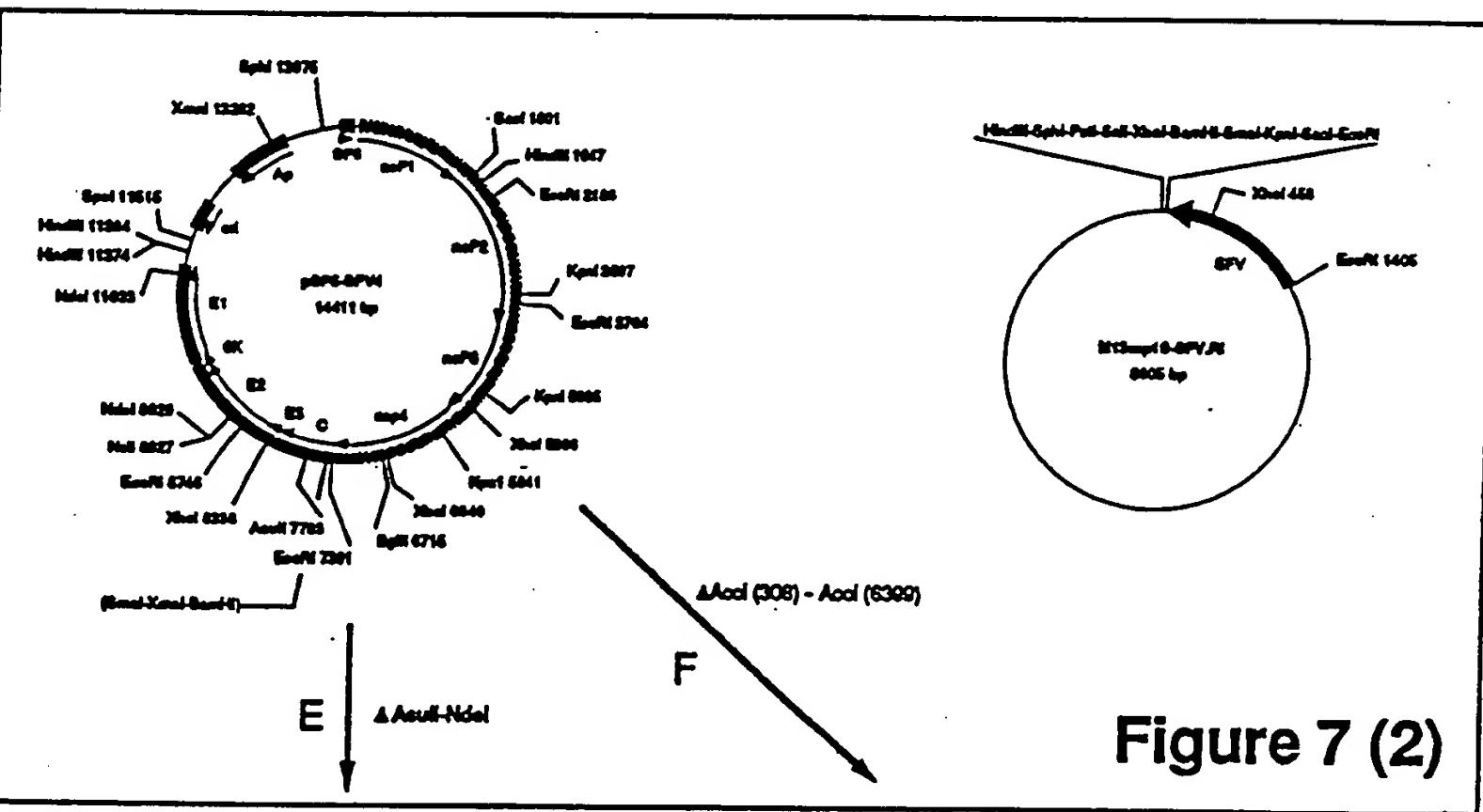
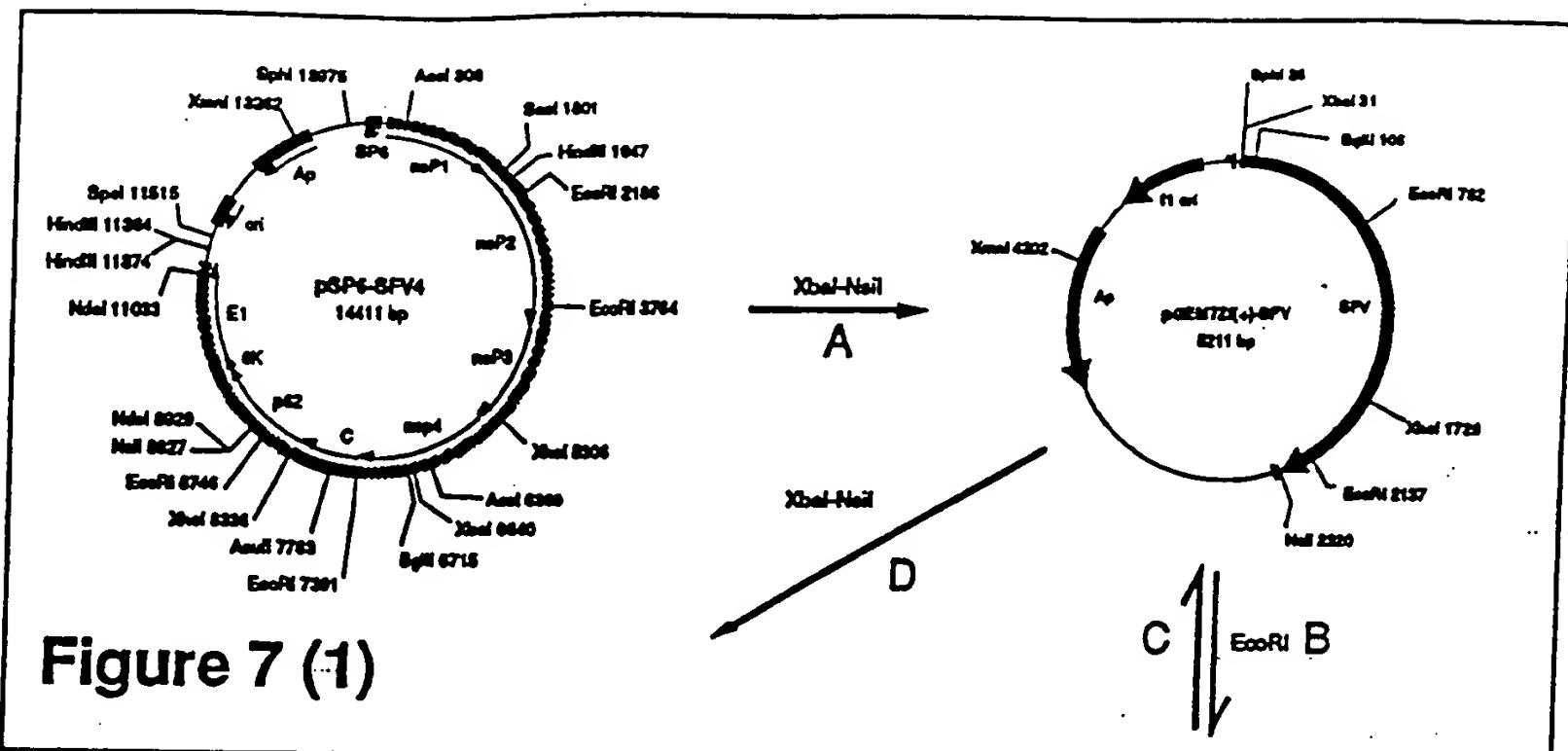
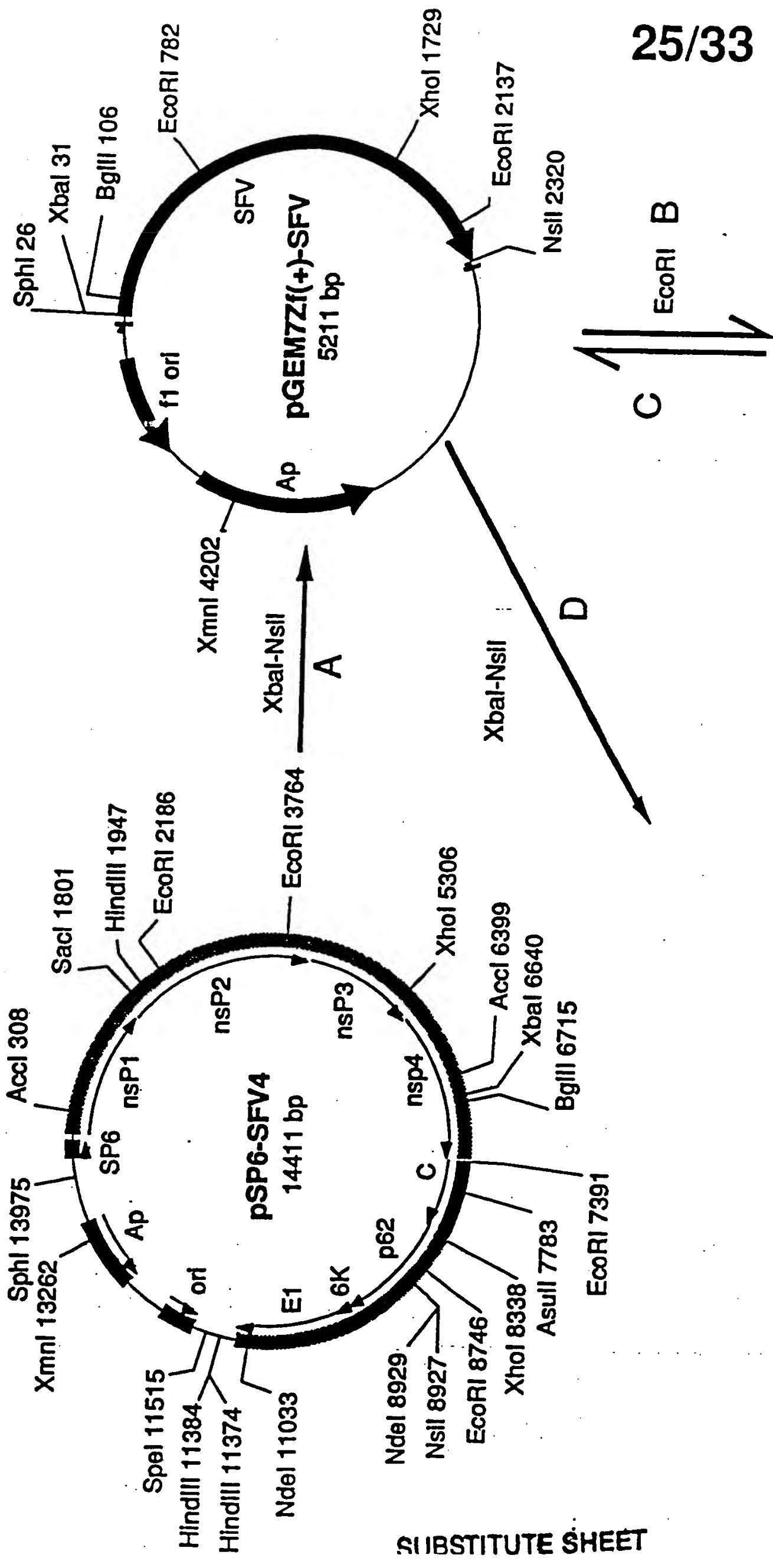


Figure 7 layout scheme

Figure 7 (1)



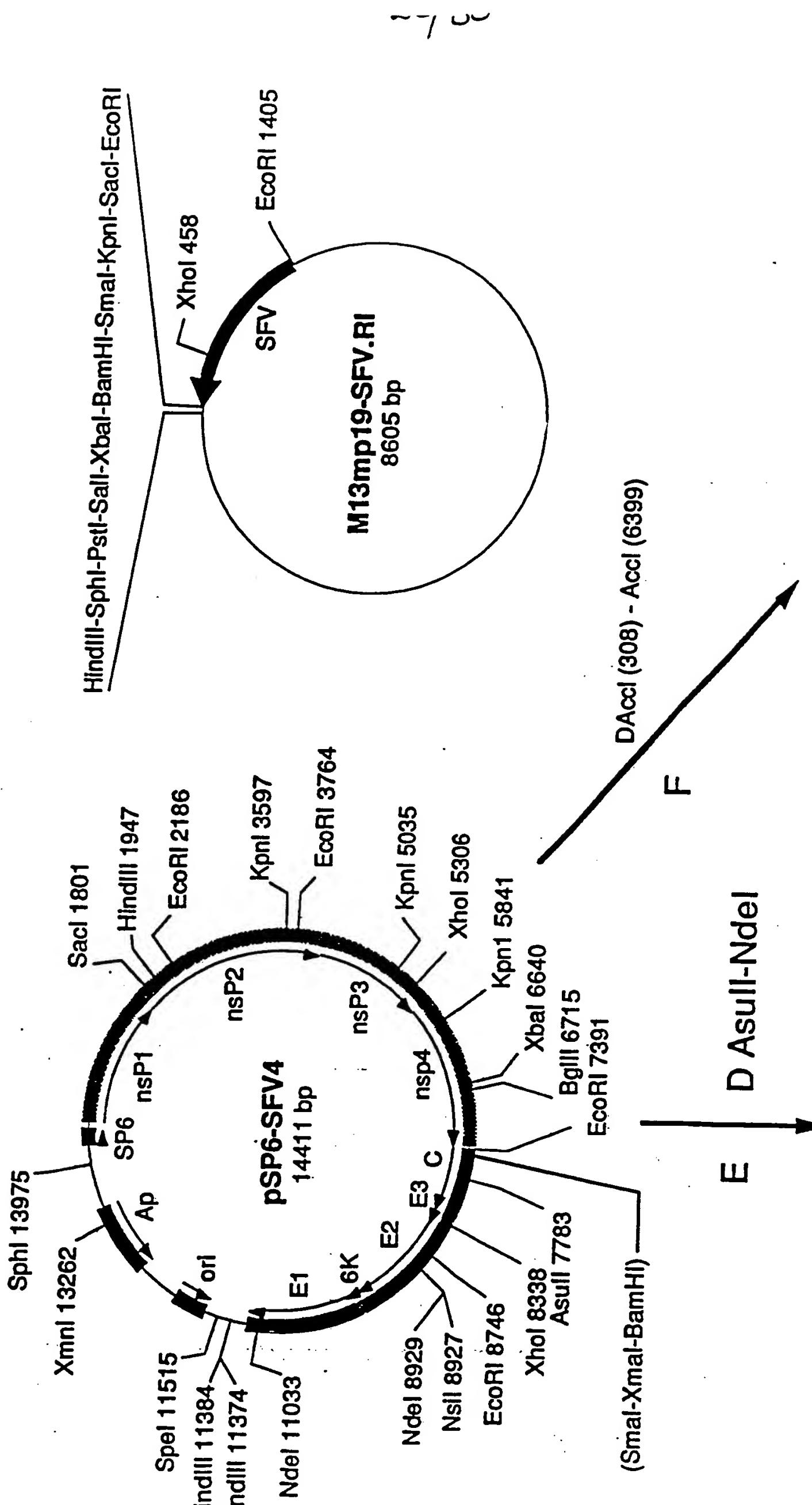


Figure 7 (2)

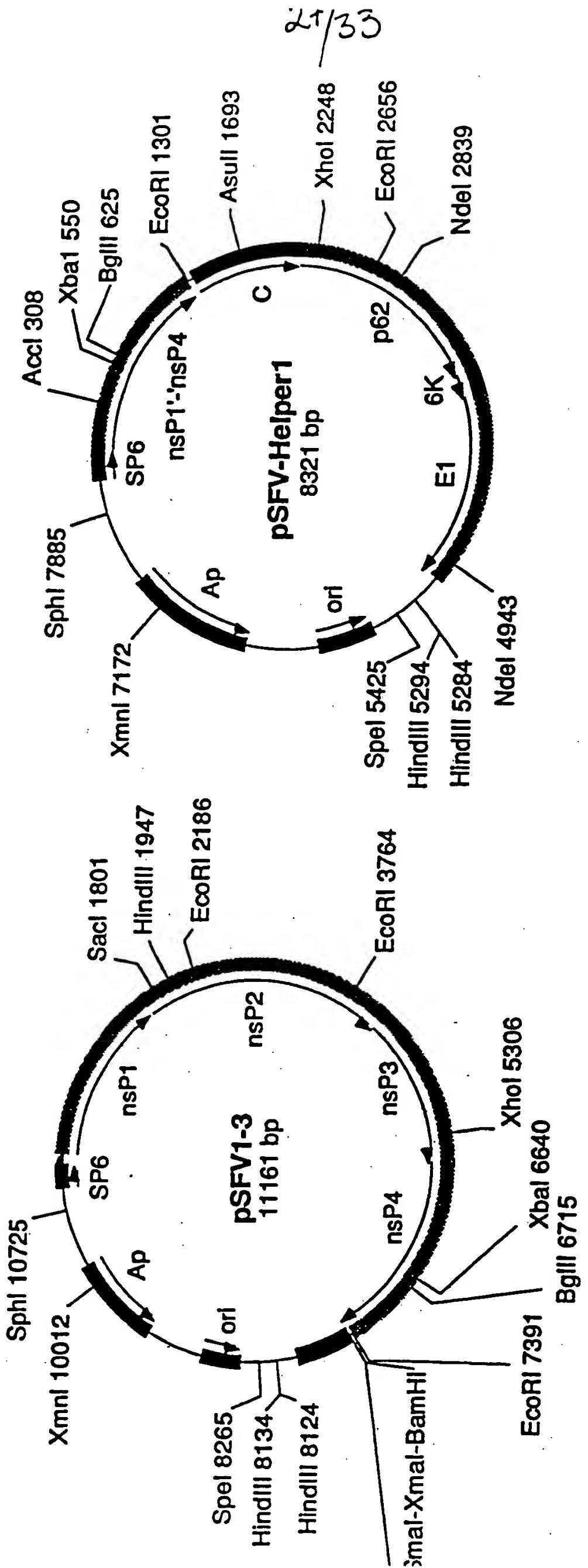
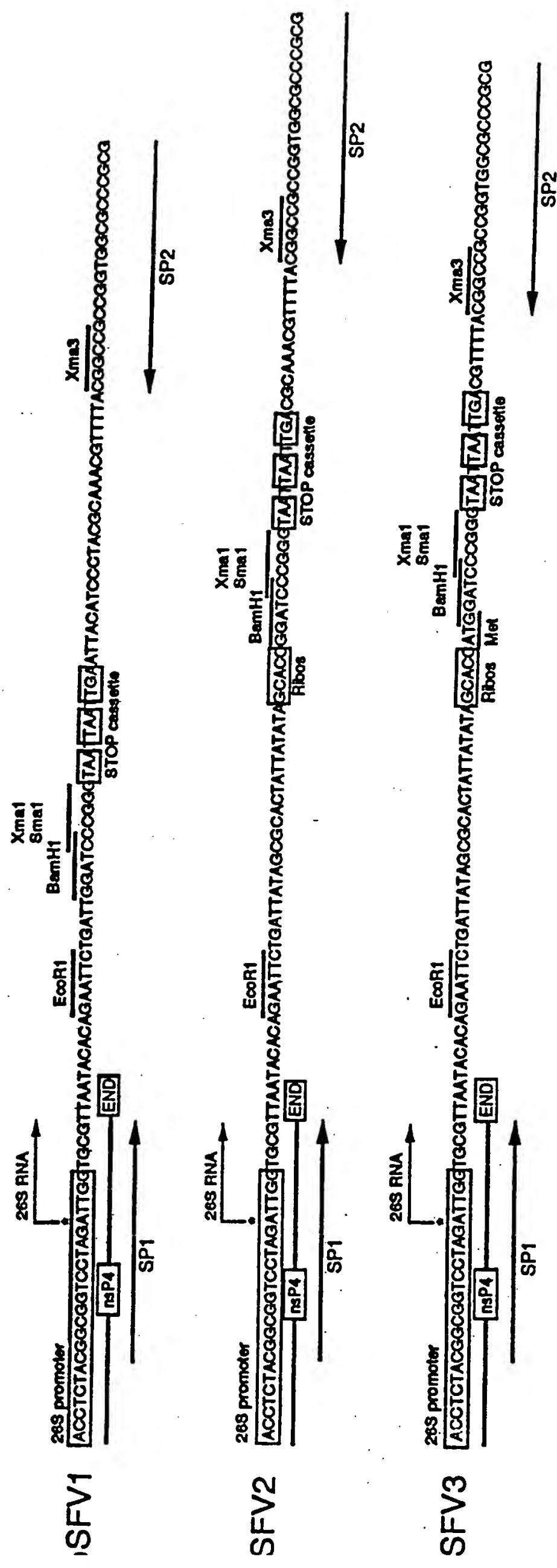


Figure 7 (3)

**Figure 8**

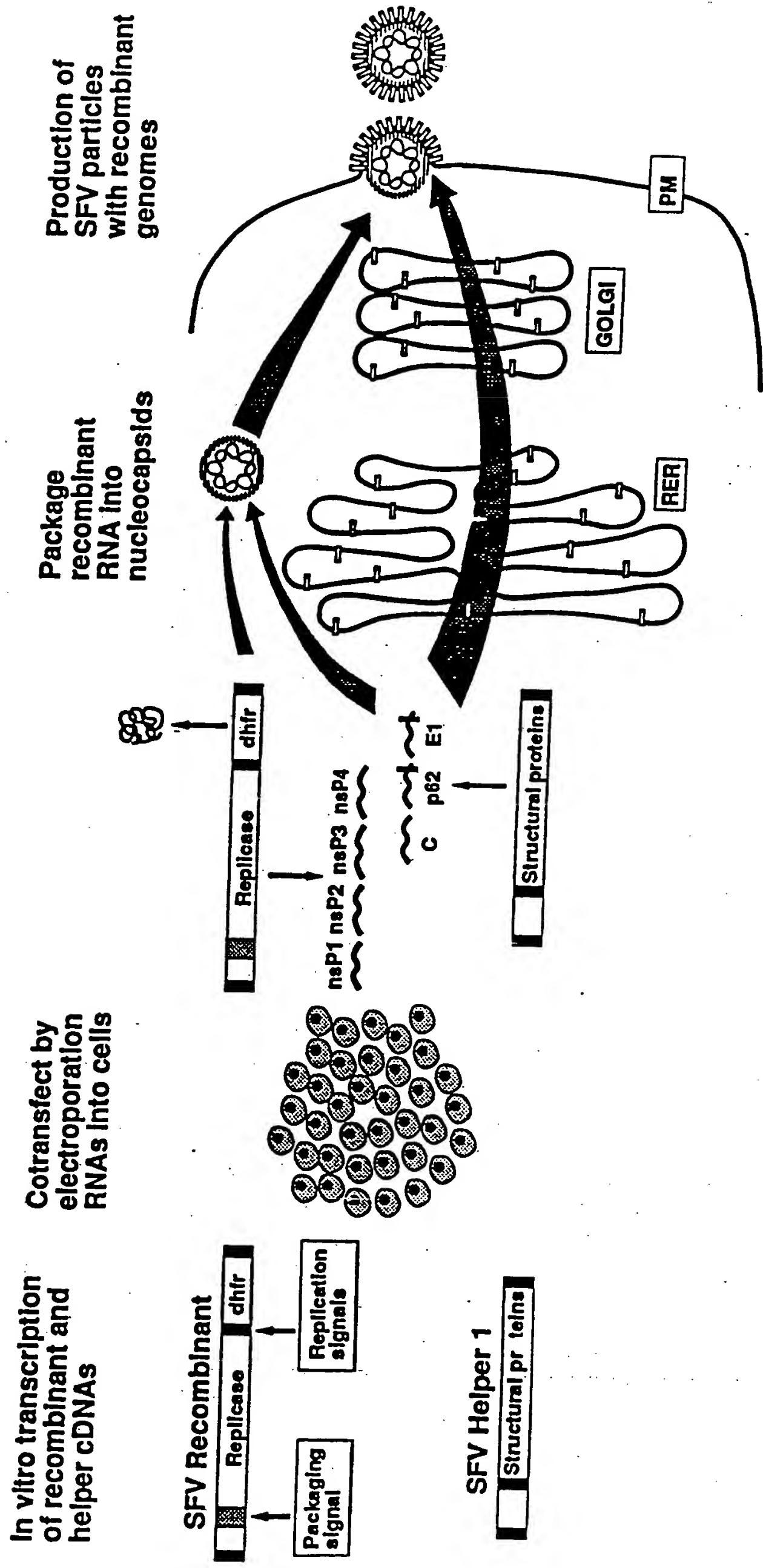
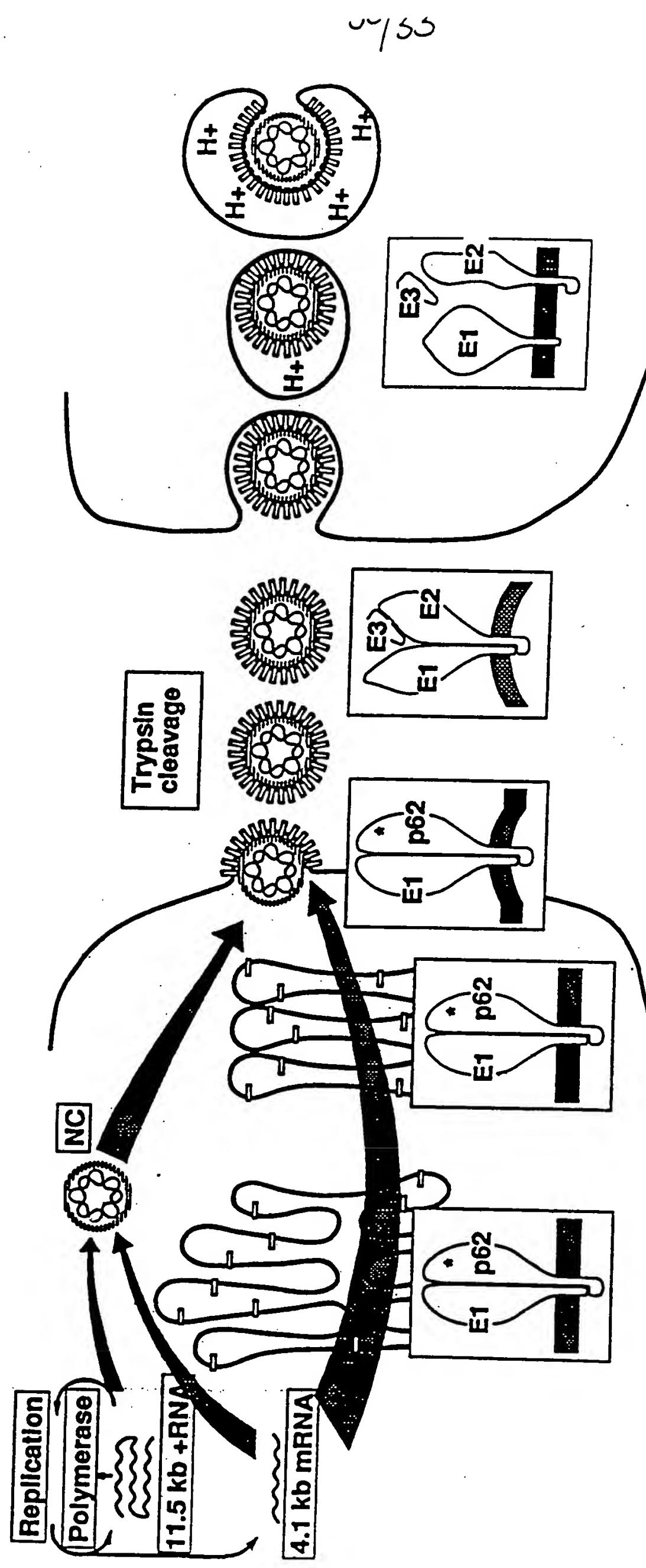


Figure 9

Figure 10



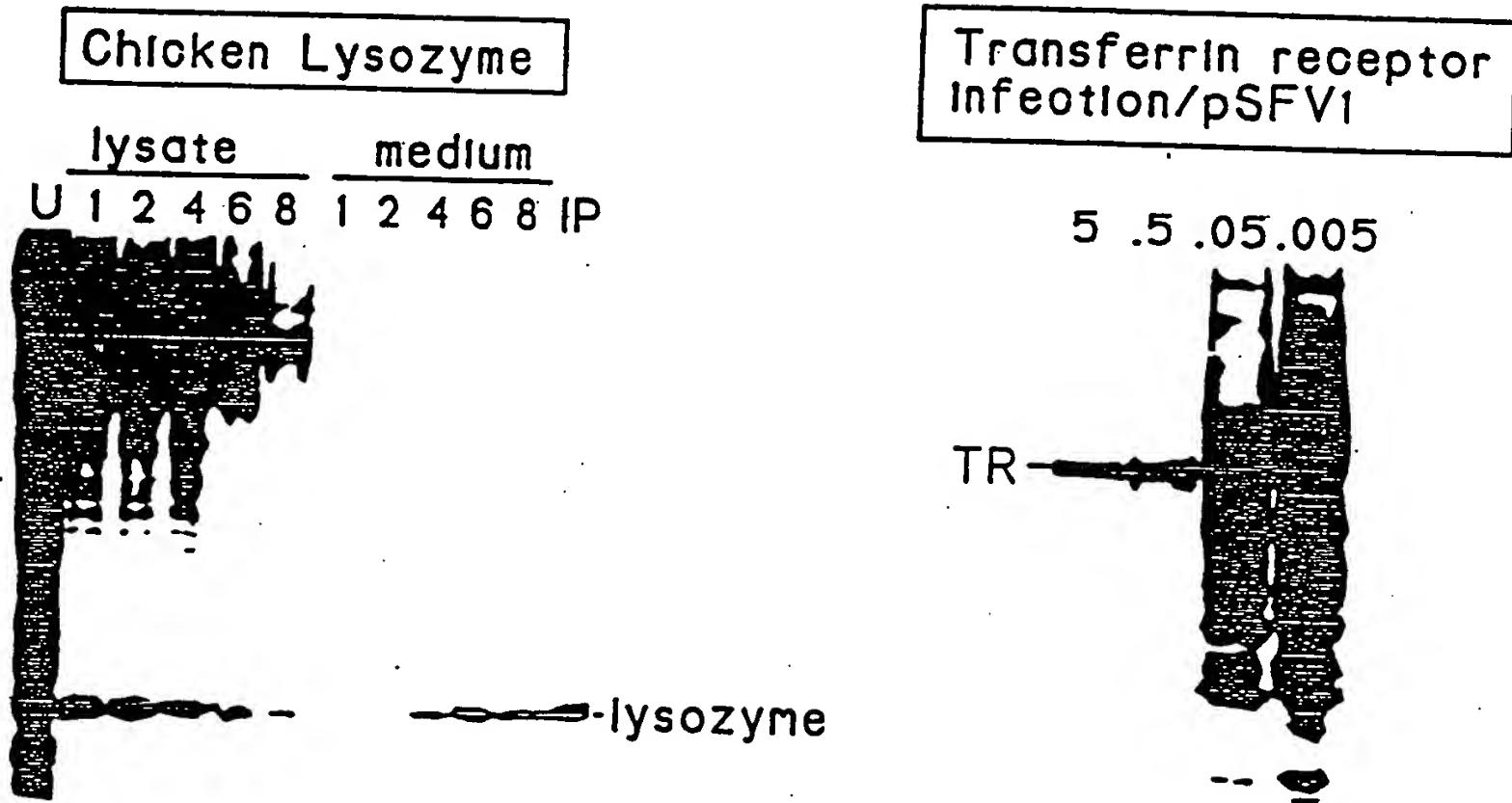
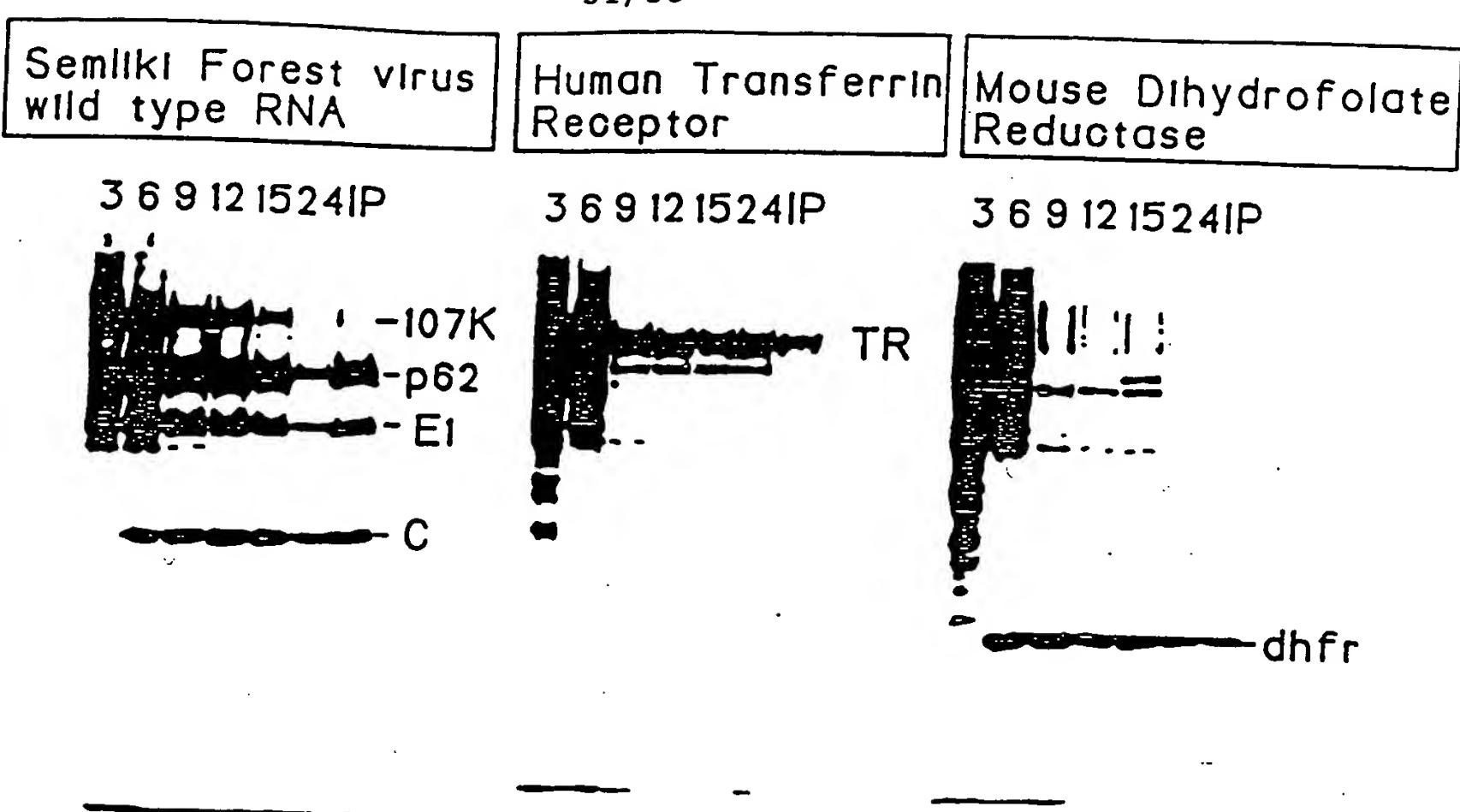


Figure 11

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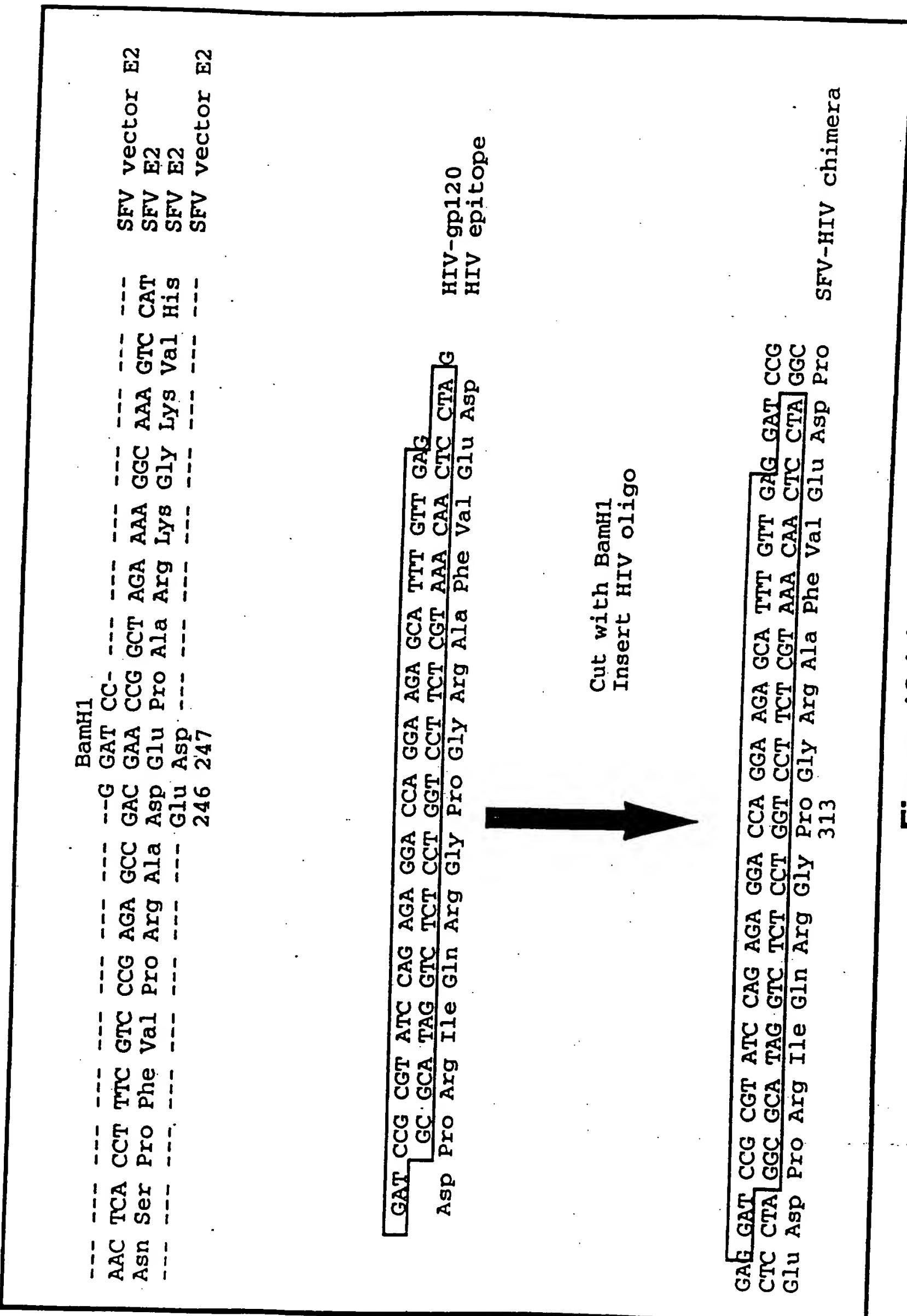


Figure 12 (1)

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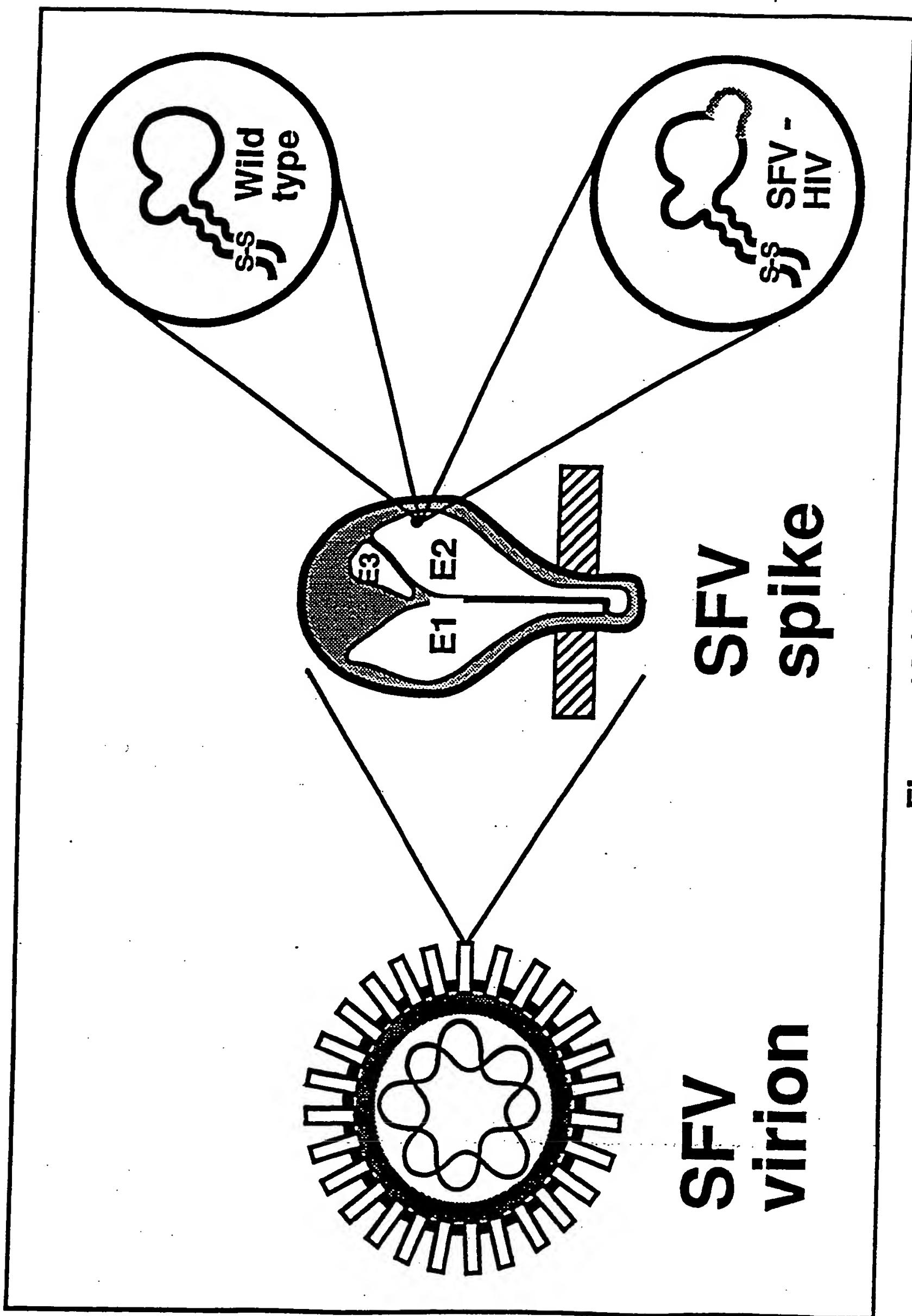


Figure 12 (2)

II. NATIONAL SEARCH REPORT

International Application No PCT/SE 91/00855

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC
 IPC5: C 12 N 15/86, C 12 N 7/01, A 61 K 39/12

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
IPC5	C 12 N; A 61 K

Documentation Searched other than Minimum Documentation
 to the Extent that such Documents are Included in Fields Searched⁸

SE, DK, FI, NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	citation of Document ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	PROC.NATL.ACAD.SCI., Vol. 84, 1987 (USA) Robin Levis et al: "Engineered defective interfering RNAs of Sindbis virus express bacterial chloramphenicol acetyltransferase in avian cells", see page 4811 - page 4815 especially page 4811, column 1 lines 1-4, 12-15, 46-48; column 2 lines 25-28 and page 4812 column 1 lines 24-26 column 2 lines 14-16	1-2, 5, 9-11, 20-22, 29, 30
Y	WO, A1, 8912095 (APPLIED BIOTECHNOLOGY, INC.) 14 December 1989, see the whole document	3, 4, 6-8, 12-19, 23-28, 31-41
Y	---	3, 4, 6-8, 12-19, 23-28, 31-41

¹⁰ Special categories of cited documents:

- ^A document defining the general state of the art which is not considered to be of particular relevance
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^T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

^X document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

^Y document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

[&] document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

17th March 1992

Date of Mailing of this International Search Report

1992-03-19

International Searching Authority

Signature of Authorized Officer

Carolina Palmcrantz

SWEDISH PATENT OFFICE

III. DOCUMENTS CONSIDERED TO BE RELEVANT		(CONTINUED FROM THE SECOND SHEET)
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Dialog Information Services, file 154, MEDLINE 85-91/July, Dialog accession no. 06757425, Medline accession no. 89059425, "Alphaviruses a new vector expressing heterologous genes", Vopr Virusol Jul-Aug 1988, 33 (4) p 502-4 -----	1-2

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 91/00855

This annex lists the patent family members relating to the patent documents cited in the above-mentioned International search report.
The members are as contained in the Swedish Patent Office EDP file on **28/02/92**
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 8912095	89-12-14	AU-D- 3775789	90-01-05